# Mapping and restructuring of an *Ae. kotschyi* derived translocation segment in common wheat

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# Declaration

**By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.**

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# Abstract

The wild relatives are an important source of new genes for the genetic improvement of wheat. At Stellenbosch University the leaf and stripe rust resistance genes *Lr54* and *Yr37* were transferred from *Aegilops kotschyi* to chromosome 2DL of wheat. In an attempt to reduce the size of the whole-arm translocation on which the resistance genes occur, homoeologous pairing was induced between the wheat and corresponding *Ae. kotschyi* chromatin. The purpose of this study was to: (i) Evaluate the testcross progeny thus obtained; identify translocation recombinants that retained *Lr54/Yr37* and to characterize these using molecular markers (ii) Test for the presence of genes for photoperiod insensitivity (*Ppd*) and reduced height (*Rht*) believed to be associated with the translocation (iii) Develop a SCAR marker for the most useful recombinant that could be recovered.

Ten putative translocation recombinants were identified following the screening of 159 hemizygous testcross $F_1$ plants with three microsatellite markers specific for chromosome arm 2DL. The recombinants were then characterized with another five microsatellite markers. Using the eight microsatellite markers the recombinants were ordered in two size categories with recombinant #74 being the shortest and having retained only proximal alien chromatin on 2DL. In addition to microsatellite markers, RAPDs, RGAs, AFLPs and SCAR markers were genetically mapped to the translocation and further resolved the recombinants into three size categories. In an attempt to find suitable markers linked to the shortest recombinant (#74) a polymorphic 410 bp AFLP fragment produced with the enzyme/selective nucleotide combination *Eco*RI – AAC/*Mse*I – CAT, was converted into a dominant SCAR marker. In addition three microsatellite markers that mapped to recombinant #74 provided a useful recessive molecular marker system to detect *Lr54/Yr37.* Evaluation of the 10 recombinants with four 2DS-specific microsatellite markers revealed a large deletion of this chromosome arm in recombinant #74. This deletion may affect plant phenotypic characteristics and a strategy to replace the deleted region in recombinant #74 is proposed.

To test for the presence of a gene for photoperiod insensitivity on the translocation, translocation-carriers plus controls were subjected to long and short day treatments, and the effect on time to flowering was studied. However, no evidence was found for the presence of such a gene. A height experiment to test for the presence of an *Rht* gene on the translocation confirmed its presence. This gene (designated *H*) appeared to be different from *Rht8* on chromosome 2DS and was mapped on 2DL. While *H* does not occur in a chromosome region that corresponds with the location of *Rht8*, it does not rule out the possibility that they could be orthologous loci. Plant height data obtained for recombinant #74 suggested that *H* was lost through recombination in this particular recombinant. A greenhouse experiment suggested that the full-length translocation increased 100 kernel mass but had a detrimental effect on overall plant yield. Since a much shorter recombinant (#74) has been obtained, this will also have to be evaluated for associated effects. Such an evaluation needs to be done under commercial growing conditions and should involve the comparison of near-isogenic bulks with and without recombinant chromosome #74.

The stripe rust resistance gene (*Yr37*) was mapped by screening hemizygous $TF_2$ progeny of the 10 recombinants with *Puccinia striiformis* pathotype 6E22A+. Recombinant #74 retained both *Lr54* and *Yr37* and the two genes therefore occur towards the centromere.

# Opsomming

Wilde verwante spesies is ‘n belangrike bron van nuwe gene vir die genetiese verbetering van koring. By die Universiteit van Stellenbosch is die blaar-roes en streep-roes weerstandsgene *Lr54* en *Yr37* vanaf *Aegilops kotschyi* na chromosoom 2DL van koring oorgedra. ‘n Poging is vervolgens aangewend om die vol-arm-translokasie waarop die weerstandsgene voorkom te verklein deur homoeoloë paring tussen die koring en ooreenstemmende *Ae. kotschyi* chromatien te induseer. Die doelstelling van hierdie studie was daarom as volg: (a) Evaluering van die verkreë toetskruis-nageslag asook die identifisering en karakterisering van translokasie rekombinante wat *Lr54/Yr37* behou het. (b) Toetsing vir fotoperiode onsensitiwiteits- (*Ppd*) en verkorte plant-hoogte (*Rht*) gene wat moontlik op die translokasie kon voorkom. (c) Die ontwikkeling van ‘n volgorde-spesifieke polimerase kettingreaksie (PKR) vir die mees bruikbare rekombinant.

Tien translokasie rekombinante is geïdentifiseer nadat 159 hemisigotiese toetskruis $F_1$-plante met drie mikrosatelliet-merkers, spesifiek vir chromosoom-arm 2DL, ge-evalueer is. Die rekombinante is hierna met vyf verdere mikrosatelliet-merkers getoets. Die data van die agt mikrosatelliet-loci het die rekombinante in twee grootte-kategorieë geplaas waarvan rekombinant #74 die kortste was met slegs die proksimale gedeelte van 2DL wat uit vreemde chromatien bestaan. Behalwe mikrosatellite-merkers is toevallig-geamplifiseerde polimorfiese DNS (RAPD), weerstandsgeen-analoog (RGA), geamplifiseerde volgordelengte polimorfisme (AFLP) en volgorde-gekarakteriseerde geamplifiseerde-streke (SCAR) merkers ook geneties op die translokasie gekarteer. Data van die addisionele merkers het dit moontlik gemaak om die rekombinante in drie grootte-kategorieë te skei. Pogings om ‘n merker vir die kortse rekombinant (#74) te vind, het gelei tot die omskakeling van ‘n 410 bp polimorfiese AFLP-fragment (geproduseer met die ensiem/selektiewe-nukleotied kombinasie *Eco*RI - AAC/*Mse*I - CAT), na ‘n dominante, volgorde-spesifieke PKR-merker. Hierbenewens kan drie mikrosatelliet-merkers wat op rekombinant #74 karteer as resessiewe merkers vir die identifisering van *Lr54/Yr37* gebruik word. Die evaluering van die 10 rekombinante met vier chromosoom 2DS-spesifieke mikrosatelliet-merkers het ‘n groot delesie van chromosoom-arm 2DS in

rekombinant #74 uitgewys. Die delesie mag plant fenotipiese kenmerke beïnvloed en daarom is ‘n strategie vir die vervanging daarvan in rekombinant #74 voorgestel.

Ten einde te toets of ‘n geen vir fotoperiode-onsensitiwiteit op die translokaie voorkom is translokasie-draers en kontroles aan lang- en kortdag-behandelings onderwerp en is die effek hiervan op dae-tot-blom gemeet. Geen bewyse vir so ‘n geen kon gevind word nie. ‘n Hoogte-eksperiment om te toets vir die teenwoordigheid van ‘n *Rht*-geen op die translokasie, het bevestig dat so ‘n geen wel voorkom. Die geen (voorgestelde simbool *H*) is gekarteer op 2DL en verskil oënskynlik van *Rht8* op chromosoom 2DS. Die verskillende chromosoom-ligging van *H* en *Rht8* skakel egter nie die moontlikheid dat hulle ortoloë loci mag wees uit nie. Plant-hoogte data vir rekombinant #74 het daarop gedui dat *H* nie meer in hierdie rekombinant voorkom nie. Data van ‘n glashuis-eksperiment het daarop gedui dat die vollengte-translokasie 100-korrel-massa verhoog maar dat dit plant-opbrengs verlaag. Aangesien ‘n aansienlike korter rekombinant (#74) verkry is, sal dit ook vir gekoppelde effekte getoets moet word. So ‘n evaluering moet egter onder kommersiële toestande gedoen word met gebruik van naby isogeniese-lyne met en sonder rekombinante chromosoom #74.

Die streep-roes weerstandgeen (*Yr37*) is gekarteer deur hemisigotiese $TF_2$-nageslag van die 10 rekombinante te toets vir weerstand teen *Puccinia striiformis* patotipe 6E22A+. Rekombinant #74 het beide *Lr54* en *Yr37* behou en die twee gene karteer dus naby die sentromeer.

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# List of General Abbreviations

| | |
|---|---|
| A | Adenine |
| *Ae* | *Aegilops* |
| ABI | Applied Biosystems |
| AFLP | amplified fragment length polymorphism |
| *Ag* | *Agropyron* |
| avg | average |
| BAC | bacterial artificial chromosome |
| BP | before present |
| bp | base pairs |
| C | Cytosine |
| cDNA | complimentary DNA |
| CS | Chinese Spring |
| CSDT | Chinese Spring Ditelosomic |
| CSN/T | Chinese Spring Nullisomic/Tetrasomic |
| CS-S | Chinese Spring Short |
| $dH_2O$ | distilled water |
| DIG | digoxigenin |
| dNTP | deoxynucleotidetriphosphate |
| eds | editors |
| EST | expressed sequence tag |
| *et al.* | et alii (Latin: and others) |
| *etc* | et cetera (Latin: and so forth) |
| FAO | Food and Agriculture Organization of the United Nations |
| Fig | figure |
| $F_n$ | $n^{th}$ generation |
| G | Guanine |
| GA | gibberellic acid |
| gDNA | genomic DNA |
| Hemi | hemizygous |
| IPTG | Isopropyl B-D-thiogalactoside |
| ITS | internal transcribed spacer |
| ITMI | International Triticeae Mapping Initiative |

| | |
|---|---|
| kbp | kilo base pairs |
| L | long arm of chromosome |
| LB | Luria Bertani |
| LRR | leucine rich repeat |
| LZ | leucine zipper |
| MAS | marker assisted selection |
| NBS | nucleotide binding site |
| *P* | *Puccinia* |
| PCR | polymerase chain reaction |
| PIC | polymorphic information content |
| pp | pages |
| QTL | quantitative trait loci |
| RAPD | randomly amplified polymorphic DNA |
| rec | recombinant |
| RFLP | restriction fragment length polymorphism |
| RGA | resistance gene analog |
| RGAP | resistance gene analog polymorphism |
| rRNA | ribosomal RNA |
| S | short arm of chromosome |
| SCAR | sequence characterized amplified region |
| SNP | single nucleotide polymorphism |
| ssp | sub-species |
| SSR | simple sequence repeat |
| STS | sequence tag site |
| *T* | *Triticum* |
| T | Thiamine |
| *Taq* | *Thermus aquaticus* |
| *Th* | *Thinopyrum* |
| TIR | toll and interleukin -1 receptor |
| U | units |
| UK | United Kingdom |
| UVPrt | *Puccinia triticina* pathotypes |

# List of abbreviations of measurements

| | |
|---|---|
| °C | degrees centigrade |
| cM | centiMorgan |
| g | grams |
| Hz | hertz |
| µg | microgram |
| µg/ml | microgram per milliliter |
| µg/µl | microgram per microliter |
| µl | microliter |
| M | molar (moles per liter) |
| µM | micro-molar |
| µmol $m^{-2}s^{-1}$ | micromoles photons $m^{-2}s^{-1}$ |
| min | minutes |
| ml | milliliters |
| mM | milli-molar |
| ng | nanograms |
| % | percentage |
| rpm | revolutions per minute |
| sec | seconds |
| v/v | volume per volume |
| v/w | volume per weight |

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# Chapter 1

# LITERATURE REVIEW

## 1.1 Introduction

Before the domestication of wheat, man collected grains from wild cereals (wheat, barley, oat, rye and *Aegilops*) as part of their diet. It is therefore believed that wheat domestication occurred in the Fertile Crescent, southwestern Asia which is regarded as the centre and origin of distribution and diversity of the wild progenitors of cultivated wheat (Feldman, 2001). Using molecular markers to compare wild and domesticated varieties, it was found that the most probable site for wheat domestication was in southeastern Turkey (Heun *et al*., 1997; Őzkan *et al*., 2002; 2005). The domestication of wheat (10300-7500 BP) consisted of two periods: the cultivation of wild forms of cereal (brittle spikes) followed by the cultivation of domesticated forms (non-brittle heads) - (Feldman, 2001). Emmer was the preferred crop for early cultivation and was also the primary crop in the spread of agriculture to Europe, west Asia and the Nile Valley (Zohary and Hopf, 1988).

Cereals have been the primary crops of many civilizations and their grains are a major source of calories for humans. Wheat is a rich source of protein (8-15%) and provides more than 25% of the total protein consumed in the human diet. In addition it contains high levels of starch (60-80%) and provides more than 20% of the calories ingested by humans. The bran fraction is a good source of fiber and contains various micro-nutrients (Zohary and Hopf, 1988; Gooding and Davies, 1997). Of all the cereals, including rice, wheat is the most important food source with a forecasted global production of 655.2 million tons and a forecasted utilization of 649.4 million tons in 2009/2010 (FAO – Crop Prospects and Food Situation, July 2009 – http://www.fao.org/docrep/012/ai484e/ai484e04.htm).

With a world population expected to reach more than eight billion people in 2050 (Hoisington *et al*., 1999) and crops that are under constant threat of diseases and pests, genetically improved crops will be needed to ensure sufficient food supplies in the future. Adequate genetic diversity is required for effective crop improvement but modern day breeding and production practices have led to reduced genetic diversity (Tanksley and McCouch, 1997; Haudry *et al*., 2007). This loss of diversity may be compensated for by the introgression of novel germplasm of which commercial varieties and landraces are the most accessible. Wild relatives of wheat, having survived the pressure of natural selection, are also a good source of new genes for physiological traits, pest and disease resistance (Fedak, 1985; Jones *et al*., 1995). Foreign genes can be transferred to wheat from its closer grass relatives using wide crosses and homologous recombination. In the case of more distantly related donor species, their genome(s) might be non-homologous to that of wheat and successful integration of new traits will require special cytogenetic procedures to induce recombination.

### 1.1.1 The classification of wheat

The tribe *Triticeae* Dumort of the family *Poaceae* (*Gramineae*) includes a number of economically important cereals such as barley, rye and wheat. The tribe includes both annual and perennial forms that each prefers different latitude and temperate regions. The tribe *Triticeae* Dumort contains the subtribe *Triticinae* that consists of the following genera: *Triticum* L., *Aegilops* L., *Secale* L., *Agropyron* Gaertn., and *Haynaldia* Schur (Morris and Sears, 1967; Miller, 1987). Morris and Sears (1967) used a classification where they combined the genera *Aegilops* and *Triticum* under the single genus *Triticum* L. However, van Slageren (1994) suggested that *Aegilops* and *Triticum* should be maintained as separate genera with the genus *Triticum* including only the cultivated taxa and their closest predecessors. The classification of van Slageren (1994) will be used henceforth in this study.

### 1.1.2 The genus *Aegilops* L.

Members of this genus are annual grasses that grow in clumps and have the ability to colonize a wide range of habitats, usually together with other *Aegilops* and wild

*Triticum* species (van Slageren, 1994). *Aegilops* L. predominantly occurs in the Mediterranean-Western Asiatic regions and only exists in its wild format. *Aegilops* L. contains five sections (*Aegilops*, *Comopyrum*, *Cylindropyrum*, *Sitopsis* and *Vertebrata*) and consists of 22 species of which 10 are diploid (2n = 14), 10 are tetraploid (2n = 28) and two are hexaploid (2n = 42) (Table 1.1) - (van Slageren, 1994).

Table 1.1 Sections of the genus *Aegilops* L., the species in each section and the genome composition of each species (adapted from van Slageren, 1994).

| **Section** | **Species** | **Genome(s)** |
|---|---|---|
| *Aegilops* | *Ae. biuncialis* | U<u>M</u> |
| | *Ae. columnaris* | U<u>M</u> |
| | *Ae. geniculata* | <u>M</u>U |
| | *Ae. kotschyi* | SU |
| | *Ae. neglecta* | U<u>M</u>, U<u>M</u>N |
| | *Ae. peregrina* | SU |
| | *Ae. triuncialis* | UC |
| | *Ae. umbellulata* | U |
| *Comopyrum* | *Ae. comosa* | M |
| | *Ae. uniaristata* | N |
| *Cylindropyrum* | *Ae. caudata* | C |
| | *Ae. cylindrica* | DC |
| *Sitopsis* | *Ae. bicornis* | $S^b$ |
| | *Ae. longissima* | $S^l$ |
| | *Ae. searsii* | $S^s$ |
| | *Ae. sharonensis* | $S^l$ |
| | *Ae. speltoides* | S |
| *Vertebrata* | *Ae. crassa* | D<u>M</u>, DD<u>M</u> |
| | *Ae. juvenalis* | DMU |
| | *Ae. tauschii* | D |
| | *Ae. vavilovii* | DMS |
| | *Ae. ventricosa* | DN |

### 1.1.3 Evolution of the genus *Triticum* L.

The genus comprises diploid, tetraploid and hexaploid forms having 7, 14 and 21 pairs of chromosomes, respectively, and a basic chromosome number of x = 7 (Miller, 1987). Evolutionary events that led to the development of the polyploid forms are illustrated in Fig. 1.1

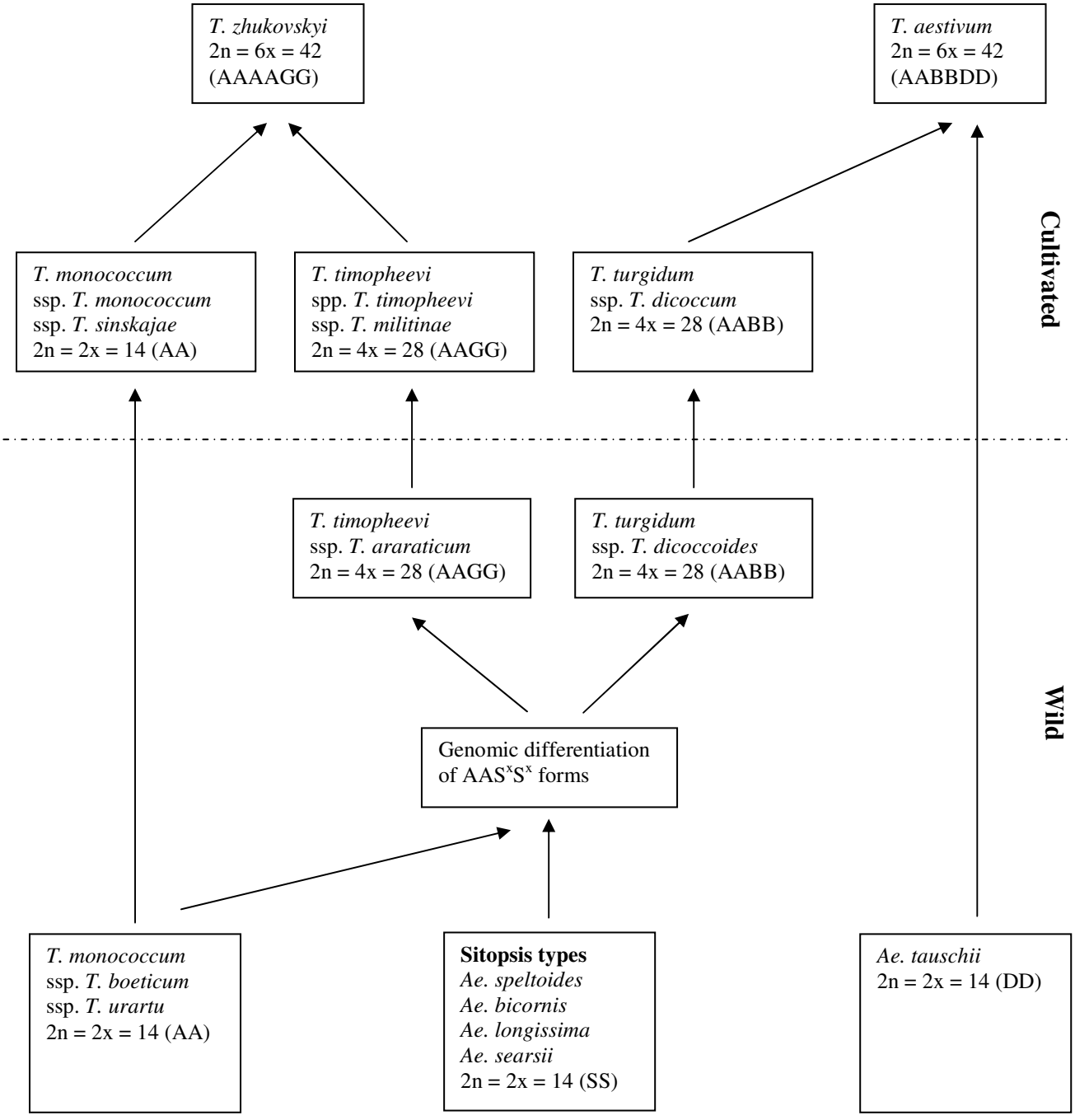



Figure 1.1 The evolutionary development of the genus *Triticum* and the origin of cultivated polyploid wheat (adapted from Miller, 1987 and Feldman *et al*., 1995).

The diploids, which include both wild and cultivated forms of *T. monococcum* and *T. urartu*, (the latter only exists in its wild form) belong to a single genomic group, AA. Presumably the two diploid species diverged from each other after a monophyletic origin which is apparent in the seed dispersal units, ecological needs and the geographical distribution of the species (Feldman *et al*. 1995). Further evidence of genomic diversification between the closely related species *T. urartu* and *T. monococcum* spp. *boeticum* was provided by Hammer *et al*. (2000) using microsatellite markers. The A-genome is furthermore common to all the polyploids in the genus *Triticum*. Tetraploid wheat includes the wild and cultivated forms of the tetraploid species *T. turgidum* (AABB) and *T. timopheevi* (AAGG) and, following allopolyploidization, gave rise to the hexaploid species *T. aestivum* (AABBDD) and *T. zhukovskyi* (AAAAGG), respectively (Fig. 1.1) (Feldman *et al*., 1995).

*T. urartu* appears to be the most probable donor of the A genome in *T. turgidum* and *T. timopheevi* (Dvořák *et al*., 1993; Jiang and Gill, 1994a; Huang *et al*., 2002; Brandolini *et al*., 2006). However, certain studies such as Zhang *et al*. (2002) found the A genome of *T. turgidum* spp. *dicoccoides* to be more closely related to *T. monococcum*. The donor of the B genome of polyploid wheat has been and still remains a source of great controversy. The following theories regarding the donor/s of the B genome have been put forward: (i) the donor is extinct, (ii) the donor is currently undiscovered, (iii) more than one parental species contributed or, (iv) the genome may have been altered since its introgression (Miller, 1987). Species of the section *Sitopsis* have previously been considered possible donors of the B and/or G genomes of the tetraploids (Kerby and Kuspira, 1988), yet Brandolini *et al*. (2006) found the A genome to be more similar to the G genome. Nuclear and cytoplasmic association studies suggested that *Ae. speltoides* is the most probable source of the B and/or G genomes (Jiang and Gill; 1994a; Wang *et al*., 1997; Khlestkina and Salina, 2001; Zhang *et al*., 2002; Sallares and Brown, 2004; Golovnina *et al*., 2007; Kilian *et al*., 2007). However, Talbert *et al*. (1995) and Blake *et al*. (1999) could not find sufficient similarity between any diploid *Sitopsis* species and the B genome of wheat which led them to conclude that the B genome had diverged from its ancestral donor species following hybridization.

The origin of tetraploid wheat is therefore either monophyletic, which comprises a single hybridization event, or di-/polyphyletic, where two or more separate hybridization events gave rise to *T. turgidum* and *T. timopheevi* (Jiang and Gill, 1994b). Using internal transcribed spacer sequences (ITS) of nuclear rRNA, Zhang *et al*. (2002) concluded that *T. turgidum* and *T. timopheevi* have a monophyletic origin or are the result of two closely followed hybridization events. However, different species-specific chromosome translocations in the Emmer and Timopheevi groups could suggest that tetraploid wheats resulted from two hybridization events involving two forms of *Ae. speltoides* and the diploid donor of the A genome (Jiang and Gill, 1994b). The latter conclusion was supported by nuclear and cytoplasmic phylogenetic analyses that grouped species containing the B and G genomes into separate clusters, with *Ae. speltoides* sharing a cluster with species having the G genome (Mori *et al*., 1995; Golovnina *et al*., 2007). In addition, estimated divergence times suggested that Emmer wheat is older than *T. timopheevi* thereby suggesting a diphyletic origin for these species (Mori *et al*. 1995; Wang *et al*., 1997).

Zohary and Feldman (1962) proposed a pivotal genome concept to explain modification of introgressed genomes following wide hybridization. They suggested that polyploid species can be cytologically divided into three clusters (A, D and U). Members of a cluster have a common unchanged (pivotal) genome and one or more differential genome/genomes which are usually altered through hybridization. They suggested that all polyploids were derived from a number of initial amphidiploids (sharing a common genome) that subsequently hybridized and differentiated. It was proposed that differentiation usually occurred only in the unshared genome/genomes while the common genome remained comparatively unaffected. Such a mechanism may explain some of the difficulty in identifying the donor species.

*Aegilops tauschii* has generally been accepted as the donor of the D genome of polyploid wheat (Talbert *et al*., 1995; Huang *et al*., 2002). Hexaploid wheat resulted from the hybridization of *T. turgidum* (AABB) and *Ae. tauschii* (DD) - (Miller, 1987). The lack of wild hexaploid wheat relatives suggested that the A and B genomes were donated by a cultivated form of tetraploid wheat. Mori *et al*. (1997) showed that *T. aestivum* is more closely related to cultivated Emmer than to wild Emmer whereas Feldman *et al*. (1995) found that the geographical distribution of *T. turgidum spp.*

*dicoccum* coincides with that of *Ae. tauschii. Aegilops tauschii* consists of two sub-species, ssp. *strangulata* and ssp. *tauschii*. Extensive research has been done to determine the specific biotype of *Ae. tauschii* which contributed the D genome. However, no conclusive results have been obtained which may suggest that multiple *Ae. tauschii* accessions contributed to the evolution of polyploid wheat (Dvořák *et al*., 1998). *Triticum zhukovskyi* originated from western Georgia and based on the karyotype, meiotic behavior, and evaluation of hybrids thereof, it was concluded that *T. zhukovskyi* was derived from the cross *T. timopheevi* and *T. monococcum* followed by chromosome doubling (Upadya and Swaminathan, 1963). This was confirmed by Dvořák *et al*. (1993) using the variation in repeated nucleotide sequences.

## 1.2 Mapping in wheat

The development of detailed wheat maps is of great importance since it elucidates the framework of agriculturally important genes and quantitative trait loci (QTL) that underlie complex genetic traits. Chromosome maps facilitate gene isolation through map based cloning and pave the way for the introgression of useful genes from related species. Genome mapping furthermore identifies molecular marker loci which, if closely linked to a gene of interest, may be used in marker assisted selection (MAS) – (Devos and Gale, 1993).

### 1.2.1 Physical mapping

Physical maps may have either a molecular or cytogenetic basis depending on the method used for their construction (Delaney *et al*., 1995a). Cytogenetic mapping constitutes the ordering of loci from an existing linkage map in relation to chromosomal cytological landmarks and includes techniques such as *in situ* hybridization, deletion mapping and the mapping of polymorphic C-bands (Werner *et al.,* 1992; Delaney *et al*., 1995a).

Once the appropriate deletion stocks have been developed, they can be employed in a simple and fast manner to physically map genes and co-dominant biochemical and DNA markers. Endo and Gill (1996) generated deletion mutants that

span the entire genome by using two alien monosomic addition lines containing, respectively, chromosomes from *Ae. cylindrica* and *Ae. triuncialis* in a Chinese Spring (CS) background. In addition to these two genotypes, they also used a translocation line in which a chromosome segment from *Ae. speltoides* had been translocated to the end of wheat chromosome arm 2BL. This translocation chromosome was associated with the gametocidal gene, *Gc1b*. Deletion mapping using molecular markers provides a practical method for constructing physical maps spanning the entire genome with the added advantage that the level of polymorphism of the markers is not important (Endo and Gill, 1996). Molecular-based physical maps on the other hand are suitable for constructing high resolution maps of small genomic areas and include long range restriction mapping and the construction of contigs (Delaney *et al*., 1995a). Cytogenetically based physical maps have been constructed for homoeologous groups 1 (Kota *et al*., 1993; Gill *et al*., 1996a), 2 (Delaney *et al*., 1995a; Röder *et al*., 1998a), 3 (Delaney *et al*., 1995b), 4 (Michelson-Young *et al*., 1995), 5 (Gill *et al*., 1996b), 6 (Weng *et al*., 2000) and 7 (Werner *et al*., 1992).

### 1.2.2 Genetic mapping

In genetic mapping the frequency of recombination is used as a measure of the relative distance between loci. Molecular linkage maps have been developed for all major crop plants and are based on linkage analysis of specially constructed mapping populations which allow the estimation of the recombination frequency between linked marker loci. However, the lack of restriction fragment length polymorphism (RFLP) in wheat (Chao *et al*., 1989) has prompted the use of wide crosses as a means to increase the level of polymorphism in mapping populations. These wide crosses include intervarietal crosses, interspecific crosses and crosses between either 'Chinese Spring' (CS) or 'Opata85' and a synthetic hexaploid (*T. turgidum* X *Ae. tauschii*). The international reference mapping population 'ITMI' (International Triticeae Mapping Initiative) constitutes such a wide cross where 'Opata85' was crossed with the synthetic line 'W7984' ('Altar84' durum X *Ae. tauschii*) – (Graingenes, http://www.wheat.pw.usda.gov/).

Extensive genetic maps of the wheat genome have been established using RFLP, AFLP (amplified fragment length polymorphism) and microsatellite (= simple

sequence repeat - SSR) markers (summarized in Varshney *et al*., 2004). Primers based on the conserved NBS-LRR (nucleotide binding site – leucine rich repeat) motifs of polypeptides involved in resistance reactions have been used in PCR (polymerase chain reaction) based strategies to isolate resistance gene analogs (RGAs). Using this technique, RGA markers have been mapped to all the wheat chromosomes (Spielmeyer *et al*., 1998; McFadden *et al*., 2006). RGA markers show close resemblance with sequences in resistance genes and have the advantage that they could either be part of a resistance gene or a marker closely linked to a resistance gene. Generally, RAPD (randomly amplified polymorphic DNA) markers have low reproducibility and may not provide reliable map data. However, RAPDs have been used to construct a genetic linkage map of einkorn wheat (Kojima *et al*., 1998). The most recently constructed linkage maps are listed in Table 1.2.

Table 1.2 A summary of recently constructed wheat linkage maps using different types of molecular markers.

| **Reference** | **Markers employed** | **Mapping population** | **Number of loci/markers mapped** |
|---|---|---|---|
| Gupta *et al*., 2002 | Microsatellites | ITMI | 66 |
| Paillard *et al*., 2003 | RFLP | Arina X Forno | 394 |
| | Microsatellite | | |
| Shi *et al*., 2003 | Microsatellites | ITMI | 1469 |
| | RFLP | | |
| Somers *et al*., 2004 | Microsatellites | Consensus map | 1235 |
| Quarrie *et al*., 2005 | RFLP | CS X SQ1 | 123 |
| | AFLP | | 194 |
| | Microsatellites | | 242 |
| Torada *et al*., 2006 | Microsatellites | Kitamoe X Münstertaler | 250 |
| | EST-SSR | | |

Although a large number of different markers were mapped in an attempt to saturate the linkage map of wheat, the D genome remains under-represented with the smallest number of RFLP and microsatellite markers mapped (Devos *et al*., 1992; Bryan *et al*., 1997; Röder *et al*., 1998b). However, *Ae. tauschii* may be used as an alternative for the genetic mapping of the D genome since it shows a high level of homology with the D genome of hexaploid wheat (Talbert *et al*., 1995; Huang *et al*., 2002) and increased levels of polymorphism (Kam-Morgan and Gill, 1989). Currently various linkage maps exist for the D genome of hexaploid wheat which have been established using molecular markers developed in *Ae. tauschii* (Pestsova *et al*., 2000; Guyomarc'h *et al*., 2002).

#### 1.2.2.1 Distribution of genes and recombination events

The integration of physical and genetic linkage maps through the joining of common markers (cytogenetic ladder map) provides insight in the physical distribution of chromosome markers and crossover positions. This allows for accurate estimation of chromosome distances and accurate molecular gene manipulation (Gill and Gill, 1994; Delaney *et al*., 1995b). The construction of composite maps for the homoeologous groups of wheat showed a high level of conservation of the linear order of loci, however, significant differences were found in the distribution of loci and distances between loci as calculated from the physical and genetic mapping experiments (Werner *et al*., 1992; Kota *et al*., 1993; Weng *et al*., 2000). This variation between genetic and physical map distances may be attributed to the irregular distribution of recombination along chromosomes which increases exponentially from the centromere towards the distal areas with recombination almost completely absent in areas surrounding the centromere (Werner *et al*., 1992; Delaney *et al*., 1995a,b; Michelson-Young *et al*., 1995). Lukaszewski and Curtis (1993) found that in chromosomes of the B genome of tetraploid wheat, recombination is almost restricted to the distal areas of the short arms. On the long arms a more even distribution of recombination that increases towards the most distal areas of the arm was evident. Recombination in the most distal 20-30% of the long arm contributed primarily to the construction of genetic maps. Physical mapping is therefore more appropriate for determining the order of loci in proximal regions compared to genetic analysis which

is more suitable for distally located loci due to the high level of recombination towards the telomeres (Werner *et al*., 1992).

Gill *et al*. (1996a,b) found that the majority of markers for the group 1 chromosomes and the long arms of the group 5 chromosomes fell into five and three clusters, respectively, separated by areas of poor marker coverage. Since the majority of markers used were cDNA clones, the distribution of markers represented the distribution of genes. Keller and Feuillet (2000) supported such a distribution of genes and estimated an average gene density of one gene every 5-20 kbp for large genome species such as barley and wheat. Erayman *et al*. (2004) estimated that 94% of genes are present in 18 major and 30 minor gene-rich regions which comprise 29% of the wheat genome. All the major gene-rich clusters occur in the most distal 35% of chromosomes. A similar result was obtained by Gill *et al*. (1993) who found that 35-46% of the distal regions of group 6 chromosomes are enriched with actively transcribed sequences whereas Gill *et al*. (1996b) found 46% of the genes to occur in the distal 25% on the long arms of group 5 chromosomes.

Ninety five percent of recombination was found to occur in gene rich clusters (Erayman *et al*., 2004). A cytogenetic ladder map of homoeologous group 4 revealed two marker-rich clusters on the long arm which accounted for more than half of the total recombination observed (Mickelson-Young *et al*., 1995) whereas recombination coincided with gene rich regions on homoeologous chromosome groups 1 and 5 (Gill *et al*., 1995a,b). Sandhu *et al*. (2001) found that 99% of recombination events on the short arm of chromosome 1B occurred in two gene-rich regions, 1S0.8 and 1S0.5. Physically, the two regions comprise approximately 14% of the chromosome arm. However, the frequency of recombination within gene rich regions is highly variable as the gene rich region 1S0.8 showed a 30X higher level of recombination compared to the gene rich region 1S0.5 (Sandhu and Gill, 2002). Significant variation in recombination between different gene-rich regions was also reported by Erayman *et al*. (2004). This was partially due to the suppression of recombination around the centromere (within the proximal 30% of wheat chromosomes). When mapping expressed sequence tags (ESTs) to chromosome deletion bins, Akhunov *et al*. (2003) found that the gene density and recombination frequency increased as the bin was

located towards distal chromosome regions. Recombination frequency was not correlated with gene density but was determined by relative chromosome position.

### 1.2.3 Chromosome pairing in wheat

During interphase the chromosomes of a somatic cell are organized in a Rabl configuration where the centromeres are grouped at the one pole of the nuclear membrane and the telomeres are spread at the opposite side of the nuclear membrane. During interphase homologous chromosome domains cognize and associate with each other, a process that primarily starts at the centromere (Schwarzacher, 1997). Prior to meiosis the centromeres of chromosomes associate in pairs. Initially the centromeres of non-homologous chromosomes associates but the level of homologous centromere association increases at the onset of meiosis. The paired centromeres are grouped into seven clusters just prior to the formation of the telomere bouquet at the beginning of meiosis. The seven centromere clusters forms tripartite structures that resolves into three paired sites whereas in tetraploid wheat elongated or V-shaped bipartite structures are formed giving rise to two paired sites (Martínez-Pérez *et al*., 1999; Martínez-Pérez *et al*., 2003). At the onset of leptotene, telomeres group to form a bouquet which results in the association of homologous telomeres. The role of the telomere bouquet remains unknown but it may be involved in synapsis or pairing of homologous (Harper *et al*., 2004). Following the formation of the telomere bouquet the homologous chromosomes start to synapse from the distal end with the middle of the chromosome the last area to synapse (Schwarzacher, 1997). Following the completion of chromosome pairing the centromeres and telomeres are dispersed.

### 1.2.4 Regulation of homologous chromosome pairing in wheat

Cultivated wheat shows strict disomic inheritance despite the presence of multiple sets of related chromosomes. Diploid-like chromosome pairing in wheat is primarily the result of the *Ph1* gene which maps to the long arm of chromosome 5B and which prevents pairing between homoeologous chromosomes (Riley and Chapman, 1958). Using X-ray irradiation, two interstitial deletion mutants of the *Ph1* gene was created in hexaploid bread wheat (*ph1b*; Sears, 1977) and in tetraploid durum wheat (*ph1c*; Giorgi, 1978).

The *Ph1* locus is located on a 2.5 MB region on chromosome 5B in the gene rich region 5L0.5 and is flanked by 5BL-1 and the distal breakpoint of the *ph1c* mutant (Sidhu *et al*., 2008). The area harbors a *cdk*-like gene cluster that consists of seven *cdk*-like genes compared to the five and two *cdk*-like gene clusters on homoeologous chromosomes 5A and 5D, respectively. The wheat *cdk* loci show close homology with the *Cdk2* locus of humans which plays a distinctive role in the regulation of meiosis (Al-Kaff *et al*., 2008). The region on 5BL contains a small area of subtelomeric DNA derived from chromosome arm 3A that got inserted in the *cdk2* gene cluster following polyploidization of wheat (Griffiths *et al*., 2006). The *cdk* locus on chromosome 5B is expressed dominantly which results in the suppression of corresponding *cdk* loci on chromosomes 5A and 5D.

Feldman (1966) studied chromosome pairing in wheat plants with increasing doses of *Ph1* and suggested that the gene exerted its effect by influencing the pre-meiotic alignment of chromosomes. Hence Vega and Feldman (1998) proposed that the primary effect of *Ph1* was through its involvement in kinetochore-microtubule interactions. The *Ph1* locus was found to have an effect on the density of centromeres with high homoeologous pairing wheat (lacking *Ph1* and *Ph2*) showing a diffuse centromere structure compared to low homoeologous pairing wheat (presence of *Ph1* and *Ph2*) that exhibited a more condensed centromere structure (Aragón-Alcaide *et al*., 1997). The latter authors suggested that the more condensed centromere may be associated with improved sister chromatid cohesion thereby avoiding disjunction at anaphase. Mikhailova *et al*. (1998) suggested that *Ph1* affects interactions between the chromatin and the nuclear matrix and between the chromatin and chromosome scaffold. This implied that chromosome remodeling may play an important role in the regulation of pairing and recombination. Colas *et al*. (2008) found that in the presence of *Ph1*, the ability to remodel chromatin depends on the degree of homology between homologues and that the lack of homology may inhibit chromosome remodeling that results in less effective pairing between homologous. However, in the absence of *Ph1*, non-homologous chromosomes do undergo remodeling and chromosome pairing occurs. Martinez-Perez *et al*. (2001) concluded that *Ph1* exhibits its effect at the somatic and meiotic level and with the primary function to ensure the specificity of centromere association rather than the initiation of centromere associations. By studying pairing frequencies of 2RL.2BL homoeo-isochromosomes in a *Ph1* and *ph1b*

background, Dvořák and Lukaszewski (2000) concluded that *Ph1* function by determining the level of chromosome homology before the onset of meiosis I rather than controlling the pre-meiotic association of centromeres.

A less effective gene for the suppression of homoeologous pairing in wheat, *Ph2*, has been identified on the short arm of chromosome 3D (Mello-Sampayo, 1968). Using X-irradiation, Sears (1982) produced a mutant (*ph2*) deficient for the terminal area of chromosome 3DS that contains *Ph2*. The effect of the *Ph2* locus on synaptic behavior is different from that of *Ph1* and affects the progression of synapses rather than actual homoeologous paring (Martinez *et al*., 2001).

In addition to *Ph* genes that suppress homoeologous recombination, a number of genes that promote homoeologous recombination have also been identified. Feldman (1966) reported genes on homoeologous chromosomes 5A and 5D that promote pre-meiotic association. Other promoters of pairing have also been identified on chromosomes 5BS, 3BL, 3DL and 2DS (Sears *et al*., 1976).

### 1.2.5 Molecular markers

Molecular markers are powerful diagnostic tools that can be used to detect polymorphism at specific loci and at genomic level and are therefore used extensively in genomic mapping and for the characterization of germplasm (Somers, 2004). In addition, molecular markers are used to 'tag' genomic regions associated with the expression of desirable traits, simple and quantitative trait loci and forms the basis for marker assisted selection (MAS). Molecular markers have an advantage over phenotypic markers since they are unaffected by environmental conditions, are more abundant and are detectable in all stages of plant development (Mohan *et al*., 1997; Gupta *et al*., 1999). Molecular markers are widely recognized for their utility in breeding and are used for the estimation of parental genetic diversity in crosses, to improve selection efficiency and to pyramid desirable genes (Marshall *et al*., 2001; Somers, 2004). Molecular markers used in plant breeding can broadly be classified as follows (Gupta *et al*., 1999):

1) Biochemical marker loci which produce an enzyme or storage protein and are visualized through biochemical assays.
2) DNA based markers which are used to identify genetic variation at the molecular level. This marker group is sub-divided into the following categories:

a) Hybridization-based DNA markers which include restriction fragment length polymorphism (RFLP) and oligonucleotide fingerprinting.
b) PCR-based DNA markers which include simple sequence repeats (SSRs), randomly amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), etc.
c) DNA chip and sequencing-based DNA markers such as single nucleotide polymorphisms (SNPs).

#### 1.2.5.1 Restriction Fragment Length Polymorphism (RFLP)

Early attempts to construct linkage maps in wheat were hampered by locus duplication, lack of polymorphism and epistatic effects as a result of polyploidy (Kam-Morgan and Gill, 1989). RFLP markers are generally not affected by polyploidy since they recognize individual loci. Other advantages of RFLPs include that they are co-dominant and could type three independent wheat homoeoloci simultaneously in a single hybridization reaction (Chao *et al*., 1989). RFLP markers were first utilized in the human genome (Botstein *et al*., 1980) whereafter it was introduced into plants (Beckman and Soller, 1986). RFLPs have been used extensively in plants and genetic linkage maps are currently available for all seven chromosomes of wheat (summarized by Varshney *et al*., 2004) and *Ae. tauschii* (Gill *et al*., 1991). Chromosome arm maps of the 21 wheat chromosomes have been established using RFLP markers (Anderson *et al*., 1992) and RFLP markers were also used in a comparative mapping study involving homoeologous group 2 chromosomes of wheat, rye and barley (Devos *et al*., 1993).

RFLP analysis constitutes the extraction of DNA, restriction digestion of the DNA and size separation of the restriction fragments by agarose gel electrophoresis. The fragments are transferred to a nitrocellulose filter and individual fragments are selected by hybridization with a labeled DNA probe (radioactive) that preferably

contains single copy DNA in the case of gDNA clones. Autoradiography is used to visualize the fragments that hybridize to the probe (Beckman and Soller., 1986; Kochert, 1994). Non-radioactive labeling techniques have been developed whereby the labeled probe is detected directly or indirectly through colorimetric, chemiluminescent, bioluminescent or fluorescent methods (Mansfield *et al*, 1995) – (Fig. 1.2).

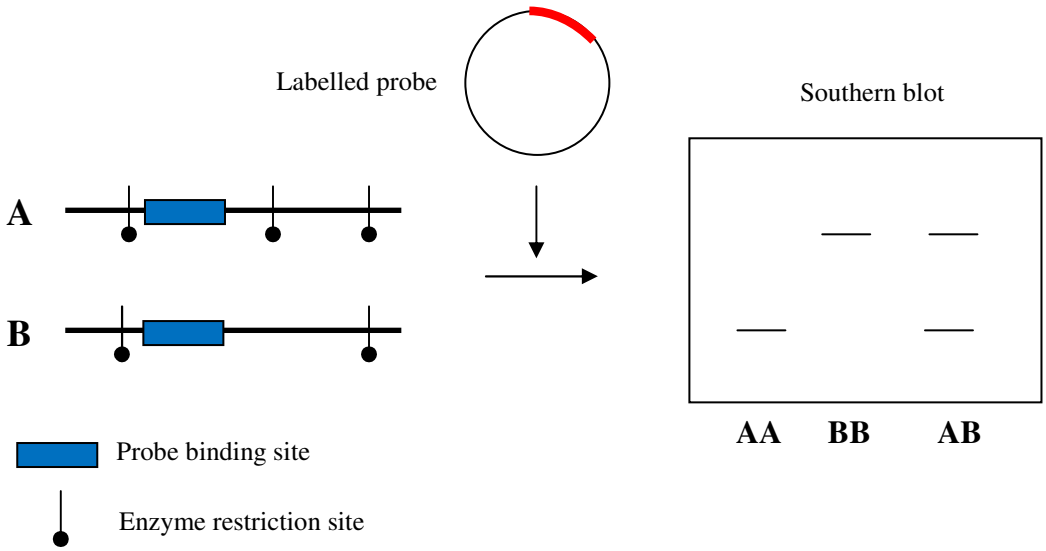



Figure 1.2 The steps involved in RFLP analysis.

RFLP polymorphisms are the result of nucleotide changes in restriction recognition sites, rearrangements due to insertions and deletions in-between restriction sites and the introgression of alleles from associated species during the evolution of hexaploid wheat (Kam- Morgan and Gill, 1989; Graner *et al*., 1990; Kochert, 1994). The positive correlation between the effectiveness of a restriction enzyme and the length of restriction fragments detected by various probes; as well as the inability of multiple enzymes to improve the likelihood of a probe to distinguish between varieties led Graner *et al*. (1990) to conclude that polymorphism in barley is mostly the result of insertion and/or deletion events. The level of polymorphism detected is influenced by the enzyme used to digest genomic DNA. Chao *et al*. (1989) found restriction enzymes with an A-T recognition sequence to be more polymorphic in wheat whereas Graner *et al*. (1990) found restriction enzymes recognizing CpG or CpXpG motifs to be less efficient due to incomplete DNA digestion. However, the level of polymorphism detected by a restriction enzyme may vary between populations and

cultivars. Using the restriction enzyme *Eco*RV, Graner *et al*. (1990) detected the highest level of polymorphism in barley whereas Devos *et al*. (1992) found *Eco*RV to reveal the lowest level of polymorphism in wheat. The type of probe used also has an influence on the number of polymorphism detected. Devos *et al*. (1992) detected a much higher level of polymorphism when using gDNA clones compared to cDNA clones.

A low percentage of RFLP markers maps to the D genome which also shows a low level of polymorphism (Kam-Morgan and Gill, 1989; Chao *et al*., 1989; Devos *et al*., 1992). However, accessions of *Ae. tauschii* showed high levels of polymorphism and therefore provide an alternative for the construction of genetic linkage maps for chromosomes of the D-genome (Kam-Morgan and Gill, 1989).

#### 1.2.5.2 Microsatellite markers

Microsatellite markers were first developed in mammals and are ubiquitous and randomly distributed in the genomes of all eukaryotes (Litt and Luty, 1989; Weber and May, 1989). The development of microsatellite markers in plants was less rapid and occurred at frequencies which are ten times less when compared to humans (Powell *et al*., 1996). Restriction fragment length polymorphism (RFLP) markers were the first markers used in the genetic mapping of hexaploid wheat. However, RFLP markers showed a low level of variation (Chao *et al*., 1989; Kam-Morgan *et al*., 1989) and infrequent distribution on genetic maps when used in bread wheat. The study of microsatellites in wheat showed higher levels of polymorphism, informativeness, genome and locus specificity (Bryan *et al*., 1997; Röder *et al*., 1998a; 1998b). In addition, microsatellite analysis requires small quantities of DNA and is easily automated which makes it suitable for the mapping of important traits and the screening of large plant populations (Röder *et al*., 1998b).

Microsatellite markers are sequences consisting of 1-6 oligonucleotide tandem repeats which are flanked by regions that serve as primer binding sites for PCR amplification (Guyomarc'h *et al*., 2002). In plant species the $(AT)_n$ sequence was found to be the most abundant and occurs once every 62 kbp while mono- and tetra-nucleotide repeats were the least frequent (Wang *et al*., 1994). The $(AC)_n$ and $(AG)_n$

repeat motives are the most common in the wheat genome and occur once every 292 kbp and 212 kbp, respectively. Approximately 50% of the $(AC)_n$ tandem repeats are compound repeats. The tri-nucleotide repeats, $(TCT)_n$ and $(TTG)_n$, are approximately ten times less frequent with 2.3 X $10^4$ estimated sites while tetra-nucleotide repeats are almost absent (Ma *et al*., 1996). Bryan *et al*. (1997) found that the majority (75%) of clones containing microsatellites have $(CA)_n$ and $(GA)_n$ repeats whereas Song *et al*. (2002) found 7% of clones screened to contain trinucleotide repeat motifs with 2.6% of these clones containing repeating lengths of eight or more. The number of repeats is positively correlated with the level of polymorphism (Bryan *et al*., 1997), however, no association could be found between the length of repeats and microsatellite informativeness and level of polymorphism (Ma *et al*., 1996; Guyomarc'h *et al*., 2002).

Microsatellite markers were found to be evenly distributed along the chromosome length except for chromosomes 1B, 2A, 2B, 3B, 4A, 4B, 5B and 6B (Röder *et al*., 1998b; Torada *et al*., 2006) where markers were clustered in centromeric regions; and chromosome arms 2BL and 2DS where markers were located distally (Röder *et al*., 1998a). Recent studies confirmed that microsatellite markers are concentrated in transcribed regions which make it particularly useful for studying genomic gene-rich regions (Röder *et al*., 1998a; Song *et al*., 2005). However, mono-, di- and tetra-nucleotide repeats were found to be restricted to non-coding regions. Tri-nucleotide repeats without G-C base pairs were also located in non-coding regions whereas 57% of tri-nucleotide repeats that contain G-C base pairs were found in coding regions (Wang *et al*., 1994).

In comparison to RFLP markers, the majority of microsatellite markers were found to be locus specific with only 8-20% of markers that amplified multiple orthologous and non-homoeologous loci (Röder *et al*., 1998b; Gupta *et al*., 2002; Song *et al*., 2005).The distribution of microsatellite markers across the three genomes of wheat reflects the level of polymorphism that exists for each genome. Röder *et al*. (1998b) mapped 93, 115 and 71 microsatellite markers to the A, B and D genomes of wheat, respectively; whereas Torada *et al*. (2006) used an intraspecific mapping population (Kitamoe X Münstertaler) to map 164, 185 and 114 microsatellite markers to the A, B and D genomes of wheat, respectively. In both studies fewer microsatellite

markers mapped to the D-genome of wheat. Additional microsatellite markers were therefore identified in the D-genome progenitor of hexaploid wheat, *Ae. tauschii*, and used to improve coverage of the D genome of hexaploid wheat (Pestsova *et al*., 2000; Guyomarc'h *et al*., 2002).

Variation exists regarding the transferability of microsatellite PCR primers, developed against genomic sequences, across homoeologous genomes and between related species. Bryan *et al*. (1997) found a low level of transferability of primers across homoeologous genomes and between less related species compared to a very high level of primer transferability between closely related species (Guyomarc'h *et al*., 2002). However, the level of transferability may relate to the origin of PCR primers with primers developed from cDNA having better transferability across related wheat genomes (Bryan *et al*., 1997).

#### 1.2.5.3 Resistance Gene Analogs (RGAs)

The majority of plant resistance genes cloned from dicotyledons and monocotyledons belong to a superfamily since they have structural similarities in their polypeptide motifs which include a leucine-rich repeat (LRR). In intracellular resistance polypeptide motifs the LRR is associated with a nucleotide binding site (NBS) and sometimes a leucine zipper (LZ) domain or a domain that shows homology to the Toll and interleukin -1 receptor (TIR) whereas in extracellular resistance proteins the LRR also contains a membrane-spanning region or a cytoplasmic protein kinase domain which is rare (Takken and Joosten, 2000). These conserved sequences are associated with pathogen recognition and signal transduction and have a putative function in protein-protein interactions in triggering the defense response (Spielmeyer *et al*., 1998).

Primers based on the conserved NBS-LRR motifs can be used in PCR based strategies to isolate resistance gene analogs. Seah *et al*. (1998) used primers derived from the Kinase-2a conserved amino acid motif and the NBS-LRR of the cereal cyst nematode resistance gene, *Cre3*, in wheat to isolate and clone RGAs from wheat and barley whereas Spielmeyer *et al*. (1998) used primers based on the conserved NBS-LRR regions of different resistance genes to map RGA loci to each of the seven

homoeologous groups of wheat. This same technique was used to identify RGAs on foreign chromosomes /chromosome fragments that contained useful resistance genes and were previously translocated to wheat from *Th. intermedium* and *T. ventricosum* (Seah *et al*., 2000; Jiang *et al*., 2005). Chen *et al*. (1998) also used primers based on the conserved NBS-LRR region of resistance genes to identify RGAs in rice, wheat and barley but used high resolution electrophoresis instead of normal agarose to detect a high frequency of polymorphic bands. Van der Linden *et al*. (2004) developed a technique called 'NBS profiling' which uses a single degenerate primer based on the conserved NBS motif in association with an adapter linked to a restriction enzyme site to produce markers linked to resistance genes and RGAs. Gennaro *et al*. (2009) used NBS-profiling on durum wheat-*Th. ponticum* recombinant lines and identified an expressed sequence in *Th. ponticum* (*AG15*) that is tightly linked to *Lr19* and relatively similar to *Lr1* and *Lr21*. This gene (*AG15*) encodes a coiled-coil-NBS-LRR protein motif and shows similarity with the wheat PM3A protein (Gennaro *et al*., 2009). McFadden *et al*. (2006) used the NBS regions of two divergent NBS-LRR gene sequences (*Go35* and *Yr10RGA*) to retrieve wheat and barley expressed sequence tag (EST) sequences from existing databases. Using a doubled haploid population (Cranbrook X Halbert), McFadden *et al*. (2006) mapped RGAs to linkage groups representing 18 chromosomes and by using nulli-tetrasomic lines placed RGA markers on all 21 chromosomes of wheat. The use of degenerate primers, based on sequence similarities in resistance genes between plant species have also been used to isolate and clone RGAs from other grain crops that include rice, maize and sorghum.

RGAs show close resemblance with resistance genes since they have similar sequences and map to the same genomic regions. RGA markers may therefore either be part of resistance genes or of sequences that are linked to resistance genes. Spielmeyer *et al*. (1998) found *Cre3* related RGA clones to map in the region of the *Cre3* resistance gene on chromosome arm 2DL whereas *Cre3* related RGA clones also detected homoeoloci on chromosome arms 2BL and 2DL. The 2BL locus coincided with the location of the previously mapped resistance gene, *Cre1*. Similarly Spielmeyer *et al*. (2000) found two unrelated RGA clones, *Rga5.2* and *RgaYr10*, to identify markers that are closely linked to leaf and stripe rust resistance genes that map near the end of wheat chromosome arm 1DS. In an attempt to clone leaf rust resistance gene *Lr1*, Cloutier *et al*., (2007) found the locus to consist of a cluster of

three paralogous sequences that are related to the RFLP marker *Xpsr567*. Only RGA567-5 was found to segregate with *Lr1* and was transferred to the susceptible hexaploid wheat cultivar 'Fielder'. When screening *Ae. tauschii* BACs with RFLP marker PSR567, Ling *et al*. (2003) identified two RGAs that encode proteins similar to the bacterial blight resistance gene *Xa1* of rice. These RGAs were separated by a physical distance of 10-50 kb suggesting that a small resistance gene family surrounds the *Lr1* locus. Based on the sequence polymorphisms of these RGAs, Qiu *et al*. (2007) developed three molecular markers of which one (WR003) co-segregated with *Lr1* in wheat and *Ae. tauschii*.

Resistance gene analog polymorphisms (RGAP) are regularly used for the mapping of rust resistance genes in wheat. Shi *et al*. (2001) identified 16 RGAP markers specific for *Yr9,* some of which showed sequence homology with resistance genes in rice. Yan *et al*. (2003) found 11 RGAP markers that were linked to *Yr5* and six markers that co-segregated with *Yr5*. Two of the markers, *Xwgp-17* and *Xwmp-18*, showed homology (97%) with a cloned resistance gene-like fragment from *Ae. ventricosa* which is also the source of the wheat resistance genes *Yr17*, *Lr37*, *Sr38* and *CreX*. Wen *et al*. (2008) identified five dominant RGAP markers linked to *Yr26* (0.5-2.9 cM) which they converted to STS markers. Pu *et al*. (2009) identified a stripe rust resistance gene in a local wheat breeding line P81 and designated it *YrP81*. In an attempt to map *YrP81* they used various markers and identified two dominant RGAP markers, RGA1 and RGA2, linked to *YrP81*. Using RGAP polymorphisms and SSR markers, Lin and Chen (2008) mapped two new stripe rust resistance genes, *YrExp1* and *YrExp2*, to chromosome arms 1BL and 2BL, respectively, of the hard red spring wheat cultivar 'Express', respectively. They found a total of 16 RGAP markers to be associated with the resistance of which nine and seven RGAP markers were linked to *YrExp1* and *YrExp2*, respectively.

The RGA genome scanning technique has an advantage to the use of arbitrary markers since it targets potentially useful genes. The markers are reproducible due to the longer primers used and involve non-radioactive detection methods. In addition the markers are very polymorphic (Chen *et al*., 1998).

### 1.2.5.4 Random Amplified Polymorphic DNAs (RAPDs)

Williams *et al*. (1990) developed a DNA polymorphism assay based on the PCR amplification of genomic DNA using short, arbitrary oligonucleotide primers which result in the amplification of random DNA products. RAPD markers are dominant, with co-dominant RAPD markers being the exception, and are inherited in a Mendelian fashion. Polymorphisms detected by RAPD markers normally involve the amplification of a single band in one of the parents which is absent in the other parent. These polymorphisms are the result of sequence variation in one or both primer binding sites thereby inhibiting primer binding and amplification; deletion of a primer binding site or deletions/insertions that change the size of the fragment amplified (Williams *et al*., 1990; Rafalski and Tingey, 1993). The level of polymorphism detected by RAPD markers in wheat are very low and are similar to the level of polymorphism detected by RFLP markers in wheat (Devos and Gale, 1992). However, Koebner (1995) found that digesting the DNA template prior to PCR may improve the level of RAPD polymorphism detected in wheat. This is the result of more efficient primer annealing along shorter DNA fragments and the less likely interference of a secondary DNA structure thereby making priming sites more accessible.

Devos and Gale (1992) found that amplification products obtained using random 10-mer primers arise mostly from repetitive DNA sequences. This may explain the tendency of RAPD markers to amplify in genomic regions not previously detected by RFLP markers (Kojima *et al*., 1998).

RAPD markers were used to study genetic diversity between diploid wheat genotypes (Vierling and Nguyen, 1992); in genotype and diversity studies in barley (Fernández *et al*., 2002); and as a marker system for the construction of a linkage map in Einkorn wheat (Kojima *et al*., 1998). However, the use of RAPDs to produce genetic markers for the construction of a linkage map is generally not recommended since they are sensitive to a variation in PCR conditions, amplify non-homologous loci that may vary between varieties and are unable to distinguish between homozygotes and heterozygotes (Devos and Gale, 1992). Jones *et al*. (1997) evaluated the reproducibility of RAPDs by comparing results between different labs using the

same reaction conditions and reagents. They found that RAPD markers were difficult to reproduce despite attempts to limit the factors causing variable results.

#### 1.2.5.5 Amplified Fragment Length Polymorphism (AFLP)

Vos *et al*. (1995) developed a technique based on the selective amplification of genomic restriction fragments using a limited set of generic primers. This technique has the advantage that it can be used with DNA of any origin or complexity and without any prior sequence knowledge. AFLP markers show monogenic, dominant Mendelian inheritance and polymorphisms are primarily based on the presence or absence of a single locus. Polymorphisms are mostly the result of point mutations in the nucleotide sequences recognized by restriction enzymes and selective primers (Hazen *et al*., 2002; Vuylsteke *et al*., 2007). Low frequencies of co-dominant AFLP markers were found in soybean. These were the result of internal fragment length variation caused by insertions or deletions or variation in microsatellite repeats (Maughan *et al*., 1996).

The AFLP technique involves restriction digestion of genomic DNA with a frequent and rare cutting enzyme and the ligation of adapters complementary to the restriction sites of the enzymes used. Two restriction enzymes with different cutting frequencies are used to reduce the number of fragments amplified thereby reducing the number of nucleotides needed for pre- and/or selective amplification. Use of two restriction enzymes also enables the labeling of one strand thereby preventing the occurrence of ‘doublets’. Primers with selective nucleotides at the 3' ends, complementary to the adaptors and adjacent nucleotides are used for amplifying a subset of restriction fragments. The number of selective nucleotides used (1-3 nucleotides) depends on the complexity of the genome, and the number of fragments amplified will decrease four fold with each selective nucleotide added. In complex genomes, primers containing a single selective nucleotide are used in a pre-amplification step which is followed by selective amplification. The fragments are separated on a polyacrylamide gel and visualized through autoradiography or silver staining (Vos *et al*., 1995; Vuylsteke *et al*., 2007). When primers are labeled with fluorescent dye, multifluorophore fragment analysis is done using an ABI DNA sequencer (Schwarz *et al*., 2000).

The restriction enzymes used have an influence on the number of fragments amplified; the level of polymorphism detected and the distribution of AFLP loci. Stodart *et al*. (2005) found *Eco*RI/*Mse*I primers to amplify a higher mean percentage of polymorphic fragments (94.7%) in wheat compared with *Pst*I/*Mse*I primers (85.1%) which in turn showed a higher mean polymorphic information content (PIC) value. Huang *et al*. (2000) also found a direct relationship between the number of fragments amplified and the *Eco*RI + CNN primer used with *Eco*RI + AGA and *Eco*RI + AGT producing the highest number of fragments and *Eco*RI + AAA and *Eco*RI + ATT producing the least number of fragments. Using the *Pst*I/*Mse*I methylation sensitive enzyme combination to map AFLP markers in a doubled haploid population, Semagn *et al*. (2006) found no apparent clustering of markers in heterochromatin areas surrounding the centromere. In soybean, 34% of *Eco*-AFLP markers segregated in clusters of more than seven markers whereas *Pst*-AFLP marker clusters were absent. In soybean *Eco*-AFLP markers were not affected by the level of methylation and only formed clusters when located in areas of low recombination (Young *et al*., 1999). *Pst*I enzyme combinations can therefore be used to avoid areas of low recombination that is normally associated with high methylation and repetitive sequences and thus clustering of markers (Tian *et al*., 2005). In addition the choice of restriction enzymes used is also influenced by genome composition. For AT-rich genomes the enzymes *Mse*I and *Eco*RI produce good results whereas for GC-rich genomes the enzymes *Pst*I and *Taq*I are preferred (Vuylsteke *et al*., 2007).

Using 10 primer pairs with three selective nucleotides, Hazen *et al*. (2002) identified 1098 distinct AFLP bands of which 53 (14%) were polymorphic in the ITMI population. They also found 23.2% of AFLP bands to be polymorphic for accessions of *T. aestivum*. Semagn *et al*. (2006) mapped 290 polymorphic loci on a doubled haploid mapping population using 45 AFLP primer pairs whereas Tian *et al*. (2005) identified 245 polymorphic bands among 242 wheat accessions using five polymorphic primer combinations. AFLP loci are randomly distributed in the wheat genome with the lowest number of loci that mapped to the D genome (Huang *et al*., 2000; Hazen *et al*., 2002; Semagn *et al*., 2006).

AFLP markers are useful to add to the validity of genetic diversity and population structure analysis where the population structure is unfamiliar such as in

germplasm collections (Stodart *et al*., 2005). The high level of polymorphic AFLP markers also makes this technique suitable for genetic diversity studies in wheat (Hazen *et al*., 2002; Stodart *et al*., 2005; Tian *et al*., 2005). AFLP markers are highly reproducible (Jones *et al*., 1997) and have the ability to simultaneously detect a large number of independent loci.

## 1.3 The transfer of foreign resistance genes to wheat

The polyploid nature of wheat facilitates the introgression of alien genetic material by giving it the ability to tolerate the presence or absence of whole chromosomes (Jauhar and Chibbar, 1999). Before a wide crosses program can be initiated it is necessary to screen wild species accessions to identify those with desirable traits. Wide hybridization is the first step in the transfer of target traits from wild species into a cultivated wheat background (Sharma and Gill, 1983). For the target gene to be integrated into a synthetic region in the wheat genome, homologous/homoeologous recombination must occur between the foreign donor chromosome and a related wheat chromosome. Recombination will stabilize the introgressed region and reduce the amount of associated foreign chromatin thereby limiting linkage drag.

Based on their genomic constitutions, the wild relatives of wheat may be divided into primary, secondary and tertiary gene pools (Jiang *et al*., 1994). Species of the primary gene pool share a high level of chromosome homology with cultivated wheat so that gene transfer may be achieved through direct hybridization and homologous recombination. Species in this category include landraces of *T. aestivum*, wild and cultivated forms of *T. turgidum* and diploid donor species of the A genome (*T. monococcum*) and D genome (*Ae. tauschii*) - (Friebe *et al*., 1996). The secondary gene pool consists of *Triticum* and *Aegilops* species having a single genome in common with cultivated wheat. Genes on homologous genomes of these species may be transferred through direct hybridization and crossover whereas the transfer of genes from non-homologous genomes may require special cytogenetic manipulations. The secondary gene pool includes the tetraploid species *T. timopheevi* and diploid *Aegilops* (section Sitopsis) species that have the G and S genomes, respectively. The latter genomes are related, but not fully homologous with the B genome of common

wheat, and it is therefore more difficult to transfer genes from these genomes (Jiang *et al*., 1994; Friebe *et al*., 1996). The tertiary pool constitutes diploid and polyploid species that are more distantly related to wheat and therefore have genomes that are not homologous, but rather homoeologous, to those of wheat. Gene transfer from these genomes requires special cytogenetic procedures (such as homoeologous pairing induction in the absence of *Ph1*; induction of chromosome translocations with mutagens such as ionizing radiation; centromeric breakage and reunion of univalents) (Jiang *et al*., 1994; Friebe *et al*., 1996).

### 1.3.1 Techniques for the transfer of foreign genetic material

The transfer of genetic material from related species to wheat may occur either through the induction of homoeologous pairing in hybrids or derivatives of hybrids that have alien addition chromosomes or wheat chromosome arms replaced by corresponding foreign chromosome arms (Sears, 1981).

The failure of wheat chromosomes to pair with distantly related chromosomes hinders the transfer of alien genetic material to wheat. However, techniques were developed that make it possible to induce homoeologous pairing. Lines that carry a deletion for the pairing regulating locus, *Ph1*, or that lacks chromosome 5B, are often used to induce homoeologous pairing between alien and wheat genetic material. Furthermore, homoeologous chromosome pairing can be observed during meiosis in hybrids of certain genotypes of *Ae. speltoides* and *Ae. mutica* with wheat. The ability of these genotypes to induce homoeologous pairing may either be the result of a gene(s) that suppresses the effect of *Ph1* or a gene(s) similar to those on chromosome 5A and 5B of wheat that promotes homoeologous pairing (Feldman, 1966). Chen *et al*. (1994) designated a *Ph1* inhibitor gene (*Ph1'*) in *Ae. speltoides* and transferred it to common wheat to produce a high pairing line. *Ph1'* mediated homoeologous recombination was subsequently used to transfer leaf and stripe rust resistance from *Ae. triuncalis*, *Ae. geniculata* and *Ae. umbellulata* to bread wheat (Chhuneja *et al*., 2008; Aghaee-Sarbarzeh *et al*., 2002). Dvořák *et al*. (2006) identified and named two loci (*Su1-Ph1* and *Su2-Ph1*) in *Ae. speltoides* that suppress the expression of *Ph1* in hybrids with wheat.

When gene transfer from very distantly related species is attempted, the suppression of *Ph1* may be insufficient to achieve translocation. Ionizing radiation may then be considered as an alternative. Ionizing radiation induces random translocations between wheat and target chromosomes; however, the technique is very laborious and has a low success rate (Sears, 1993). Friebe *et al*. (1993) transferred leaf rust resistance from *Ag. intermedium* to wheat following the irradiation of wheat – *Ag. intermedium* chromosome addition lines.

Another method for the introduction of foreign material involves substitution of one arm of a wheat chromosome with a corresponding foreign chromosome arm (Sears, 1981). Robertsonian translocations occur when the centromeres of univalents misdivide during ana/telophase I, whereafter the broken telocentric chromosomes are included in the same nucleus and randomly reunite during interkinesis (Friebe *et al*., 2005). This technique has been utilized to transfer the stem rust resistance gene *Sr27* from chromosome 3R of rye to each of the homoeologous group 3 chromosomes of wheat (Marais and Marais, 1994).

## 1.4 The *Ae. kotschyi* derived S14 translocation

Antonov and Marais (1996) reported the transfer of leaf rust resistance genes from a collection of wild *Triticum* and *Aegilops* species to common wheat. Following screening of the species collection with pooled *Puccinia triticina* inoculum, 127 resistant accessions were crossed successfully with Chinese Spring (CS) wheat. Seventy $F_1$ hybrids fully expressed the targeted resistance. One of the donor lines was *Ae. kotschyi* (accession 617). When tested against a wider range of *P. triticina* pathotypes (*UVPrt2*, *UVPrt3*, *UVPrt8*, *UVPrt9* and *UVPrt13*) the *Ae. kotschyi* cross derivatives showed resistance to all. Resistant progeny were therefore continuously backcrossed to CS and a disomic addition line, 8078, was subsequently selected from the $B_3F_2$. The addition chromosome was found to carry both leaf and stripe rust resistance genes derived from *Ae. kotschyi*. The addition line exhibited the tenacious glumes trait which is determined by the dominant gene, *Tg*, located on chromosome 2DS. It was therefore assumed that the addition chromosome in 8078 belonged to homoeologous group 2 (Marais *et al*., 2003; 2005).

Figure 1.3 A spike of the wild species *Aegilops kotschyi* from which leaf rust (*Lr54*) and stripe rust (*Yr37*) resistance genes were transferred to common hexaploid wheat.

Marais *et al*. (2005) made use of double monosomics in an attempt to induce centric break and fusion (Robertsonian) type translocations between the addition chromosome and homoeologous group 2 wheat chromosomes. To achieve this, they crossed the disomic addition line, 8078, with each of the CS group 2 monosomics (2A, 2B and 2D) whereafter they selected leaf rust (*UVPrt8*) resistant $F_1$ seedlings with 2n = 42 chromosomes. They testcrossed the $F_1$, which they assumed to be double monosomic for the respective wheat group 2 chromosome and the group 2 addition chromosome from *Ae. kotschyi*, with leaf rust susceptible testers. The tester genotypes were W84-17 (breeding line) and CS-S (a semidwarf, near isogenic line of CS). The resistance was transmitted to approximately 18% of the testcross progeny. These plants were selfed and the $TF_2$ families thus derived were seedling tested for leaf rust resistance to determine the segregation ratios (Fig. 1.4).

From the data it was apparent that the complete addition chromosome was transferred in most cases. They, however, found one double monosomic 2D derived $F_2$ population (named S14) with a high proportion (96%) of resistant plants, suggesting that the resistance occurred on a compensating translocated chromosome. Marais *et al*. (2005) conducted monosomic and telosomic analyses with the S14 translocation and concluded that it involved chromosome arm 2DL. The translocated region and corresponding wheat 2DL telosome did not pair during meiosis suggesting that the introgressed region occupied a large part, or all of chromosome arm 2DL. The translocation was shown to carry leaf rust (*Lr54*) and stem rust (*Yr37*) resistance genes from the *Ae. kotschyi* addition chromosome.

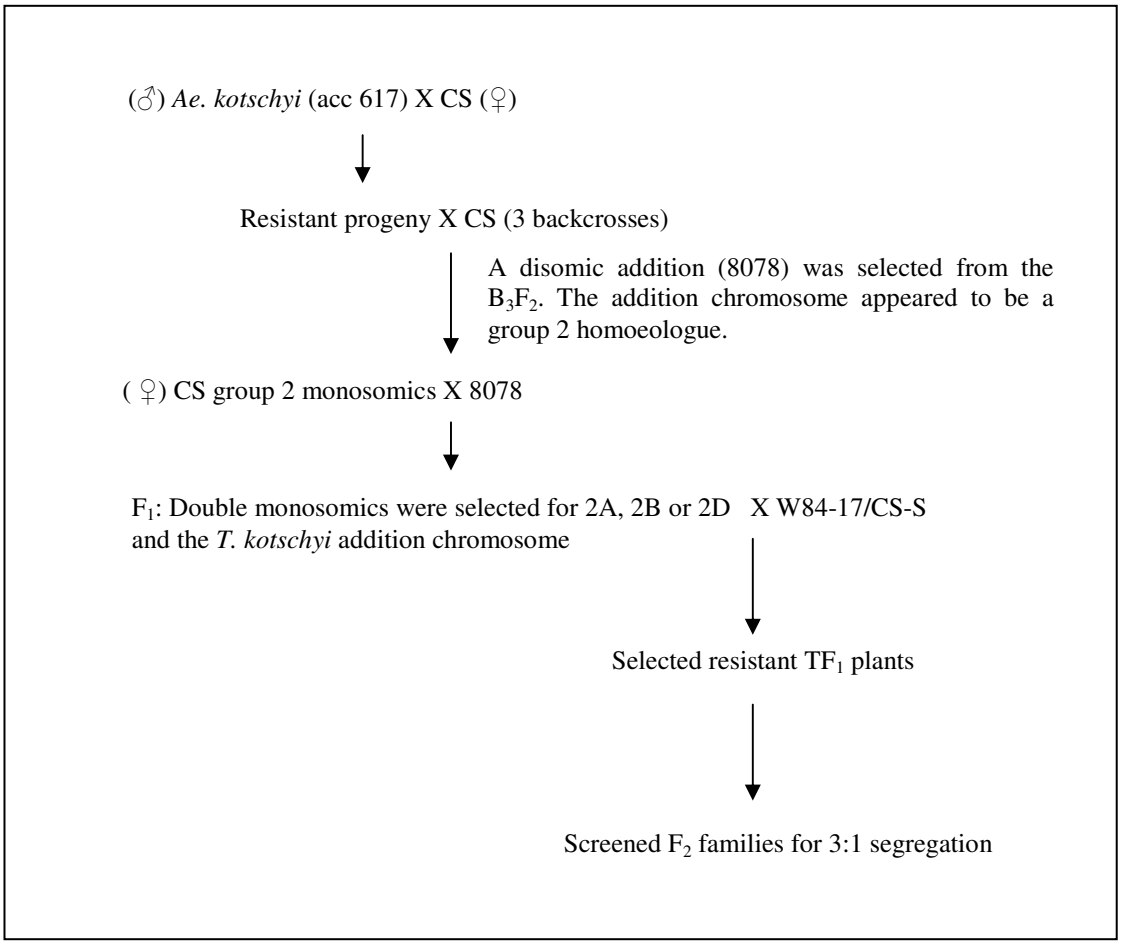



Figure 1.4 The strategy that was used to transfer leaf and stripe rust resistance genes from *Ae. kotschyi* to wheat (Marais *et al*., 2005).

Marais *et al*. (2005) found that the S14 translocation plants were shorter than CS-S and W84-17, both of which are believed to carry *Rht-B1b*. The dwarfing gene, *Rht8*, has been mapped to chromosome 2D (Komugi Catalogue of Gene Symbols – http://www.shigen.nig.ac.jp). They therefore suggested that the *Ae. kotschyi* derived translocation may contain a height reducing gene that could be homoeoallelic to *Rht8,* and which in combination with *Rht-B1b,* may produce dwarfs, *Rht-B1b(-) Rht?(-).* The S14 translocation plants were also found to flower earlier than the parents. Three photoperiod insensitive genes, *Ppd-A1*, *Ppd-B1* and *Ppd-D1*, were mapped to the homoeologous group 2 chromosomes (Welsh *et al*., 1973; Law *et al*., 1978; Scarth and Law., 1983) and Marais *et al*. (2005) therefore suggested that the translocation may also contain a gene for photoperiod insensitivity.

### 1.4.1 Homoeologous group 2 chromosome maps

Extensive genetic and physical maps are available for the homoeologous group 2 chromosomes. Such maps revealed a generally high level of co-linearity between homoeologous chromosomes 2A, 2B and 2D with the exception being chromosome arm 2BS where a translocation of the distal region occurred to chromosome arm 6BS (Devos *et al*., 1993; Nelson *et al*., 1995). Physical maps of homoeologous group 2 showed a random distribution of dinucleotide microsatellite markers along the chromosome length (Röder *et al*., 1998a). However, comparison of genetic RFLP maps of wheat, rye and barley by Devos *et al*. (1993) showed markers to be clustered in areas surrounding the centromere in wheat and rye. Marker distribution patterns reflected the rate of recombination along the chromosome length which was found to be at least ten times more frequent in distal areas compared to proximal regions (Delaney *et al*., 1995a). Similarly Röder *et al*. (1998a) found 60% of recombination to occur in the distal 20% region of chromosome 2AL, whereas the proximal 65% of chromosomes 2BL and 2DL showed reduced recombination. Comparison of map distances between homoeoloci suggested that recombination occurred more frequently on chromosome 2D than on chromosomes 2A or 2B (Devos *et al*., 1993).

## 1.5 Dwarfing genes

The control of plant height is a complex trait which is influenced by genes located on a number of different chromosomes as well as environmental factors. The major genes associated with the control of plant height may be classified as either gibberellic acid (GA) sensitive or GA insensitive depending on their response to exogenously applied gibberellins. The primary effect of these genes is a reduction in plant height which is the result of an altered growth pattern during plant development. It can therefore be expected that these genes will have pleiotropic effects on plant characters such as the hormonal regulation of GA-insensitivity, plant growth, morphology, physiology, grain yield and quality (Gale and Youssefian, 1985).

### 1.5.1 Gibberellic acid (GA) insensitive dwarfing genes

The GA insensitive dwarfing genes consist of two homoeologous allelic loci, *Rht-B1* and *Rht-D1*, which are located on chromosomes 4B and 4D, respectively. Börner *et al*. (1996) proposed new symbols for the wheat GA insensitive dwarfing genes. This nomenclature will be used henceforth whereas the nomenclature used previously is indicated in brackets. The *Rht-B1* locus consists of six alleles, *Rht-B1a* (tall), *Rht-B1b* (*Rht1*), *Rht-B1c* (*Rht3*), *Rht-B1d* (*Rht1S*), *Rht-B1e* (*Rht Krasnodari 1*) and *Rht-B1f* (*Rht T. aethiopicum*) compared to the four alleles of the *Rht-D1* locus, *Rht-D1a* (tall), *Rht-D1b* (*Rht2*), *Rht-D1c* (*Rht10*) and *Rht-D1d* (*Rht Ai-bian 1a*) - (McIntosh *et al*., 1998).

The dwarfing alleles *Rht-B1b* and *Rht-D1b*, located on chromosomes 4BS and 4DS, respectively, were derived from the cultivar 'Norin 10' which was selected from initial crosses of American varieties with the ancient Japanese cultivar 'Shirodaruma' (Gale and Youssefian, 1985). *Rht-B1b* and *Rht-D1b* are mutant alleles of the wheat height regulating genes *Rht1* and are the result of nucleotide substitutions that created translational stop codons. The latter mutations alter the N-terminal region of the proteins they encode and change *Rht-B1a/Rht-D1a* into mutant repressors that are less influenced by gibberellins than the normal protein (Peng *et al*., 1999). *Rht-B1b* and *Rht-D1b* played a vital role in the 'green revolution' and have been used extensively in plant breeding programs to reduce plant height and increase grain yield by improving lodging resistance (Gale and Youssefian, 1985). A height reduction of approx. 14-17% was found for lines carrying single dwarfing alleles *Rht-B1b* or *Rht-D1b* whereas these alleles in combination caused a 42% reduction under optimal conditions (Flintham *et al*., 1997; Worland *et al*., 1998a; Butler *et al*., 2005). The *Rht-B1c* allele on chromosome 4BS (Börner *et al*., 1997) has a more drastic effect in reducing plant height and *Rht-B1c* semi-dwarf lines showed a height reduction of 50% compared to the tall control when cultivated under optimal conditions in Germany and the UK (Flintham *et al*., 1997). This dwarfing gene, however, remains unused in commercial varieties due to its excessive effect on height reduction. *Rht-B1b*, *Rht-D1b* and *Rht-B1c* function by reducing the ability of vegetative tissues to respond to gibberellic acid. Gibberellic acid normally promotes cell elongation in the stems and leaves which results in increased plant height and leaf area. *Rht-B1b* and *Rht-D1b*

have no significant effect on cell number whereas *Rht-B1c* is associated with a reduction in the number of leaf cells (Hoogendoorn *et al*., 1990).

The reduction in plant height associated with *Rht-B1b* and *Rht-D1b* is directly correlated with an improved harvest index, an increase in tiller number and spikelet fertility thus resulting in an increased number of kernels per spike. However, the higher grain number in dwarf and semi-dwarf lines has a negative effect on grain size (Gale and Youssefian, 1985). Compared to tall lines, dwarf lines containing the *Rht-B1c* allele showed a 27% increase in grain number yet this was associated with a 23% reduction in grain size (Flintham and Gale, 1983). The increase in plant yield was the result of reallocation of dry matter to the developing spikes of semi dwarf genotypes following the reduction of stem extension growth which decreased the need of the stem for assimilates. The difference in dry matter partitioning improved floret survival which led to an increased number of receptive florets with a higher carpel biomass at anthesis (Youssefian *et al*., 1992). Pinthus and Gale (1990) concluded that the reduction in grain size and protein levels was an indirect pleiotropic effect of *Rht* alleles on several characters of various maternal tissues which influenced grain size and protein.

The grain yield advantage of dwarf and semi-dwarf varieties is subject to environmental conditions, genetic background and whether these genes occur in a winter or spring wheat background (Butler *et al*., 2005). The yield improvement associated with *Rht* alleles is more consistent in winter wheat comparisons (Gale and Youssefian, 1985) whereas dwarf and semi-dwarf lines only showed a yield advantage when cultivated in high yielding environments. No yield advantage, or even a reduction in yield, was seen when semi-dwarfs were cultivated in environments where the yield potential is low (Singh *et al*., 2001). This variation in grain yield is due to the effect that environmental stress factors, temperature and drought, have on *Rht* genotypes. When tall, dwarf and semi-dwarf spring wheat lines were evaluated for grain yield under rainfed and irrigated conditions, the dwarf and semi-dwarf lines out-yielded the tall lines under both conditions. The dwarf lines, however, showed the highest percentage of yield loss due to drought stress (Nizam Uddin and Marshall, 1989). In a similar experiment, Butler *et al*. (2005) found the yield of the dwarf lines to be significantly lower, and that of semi-dwarf lines similar

or lower, when compared to tall genotypes under both environments. The exception was the *Rht-B1b* + *Rht-D1a* class which showed a slight yield advantage under optimal conditions. Laing and Fischer (1977) found Norin-10 semi-dwarf wheat varieties to be superior to taller varieties irrespective of the conditions. Richards (1992) showed grain yield to be determined by optimum plant height (70-100cm) rather than the presence of dwarfing genes when spring wheat is cultivated in dry environments.

Following genetic analysis of the cultivar 'Saitama 27', Worland and Petrovic (1988) found that it contained a single weak GA-insensitive gene *Rht-B1d* (*Rht1S*) on chromosome 4A (re-assigned to chromosome 4B at the Seventh International Wheat Genetics Symposium, Cambridge UK, 1988) which is allelic to *Rht-B1b* and *Rht-B1c*. In varietal comparison studies with *Rht-B1b*, Worland and Petrovic (1988) found *Rht-B1d* to reduce plant height with approx. 11% combined with an increase in grain number but a reduction in grain size. The 'Saitama 27' allele is regularly combined with the GA sensitive dwarfing gene, *Rht8*, and is less prone to heat and drought stress which results in higher fertility and yield (Worland and Petrovic, 1988; Ganeva *et al*., 2005). Worland and Sayers (1995) found an allelic variant of *Rht-B1b* which they designated *Rht-B1e* (*Rht1* (*B.dw*)). This variant proved to be superior to *Rht-B1b* in reducing plant height and increasing grain yield. In addition to *Rht-D1b* and *Rht-B1b*, Cadalen *et al*. (1998) identified three further loci on chromosomes 4BS, 7AL and 7BL and an interaction between loci on chromosomes 1AS and 1BL to be associated with the regulation of plant height. Börner *et al*. (1992) also identified factors on group 5 and 7 chromosomes responsible for a reduction in gibberellic acid usage thereby making plants more insensitive.

### 1.5.2 Gibberellic acid (GA) sensitive dwarfing genes

The GA sensitive dwarfing genes in southern European wheat varieties were introduced by the Italian breeder Strampelli who used the Japanese variety 'Akakomugi' in crosses with Italian wheat varieties. The GA sensitive dwarfing genes are the preferred dwarfing genes in southern European varieties due to their ability to tolerate high temperatures (Worland *et al*., 2001).

*Rht8* is a weak height reducing gene which reduces plant height by 2-8cm and has no effects on other plant characteristics (Börner *et al*., 1993; Worland *et al*., 2001). *Rht8* is associated with the photoperiod insensitivity gene, *Ppd-D1*, and the yellow rust susceptibility allele, *yr16*, on chromosome 2D. The *Ppd-D1* gene also reduces plant height as a result of a shortened plant growth period and when combined with *Rht8*, reduces plant height with approximately 17-18 cm. Both the *Ppd-D1* and *yr16* genes reduce the number of spikelets; however, they increase spikelet fertility, number of grains per spikelet and grains developing in central florets (Worland *et al*., 1988).

Another dwarfing gene, *Rht12*, was evaluated in different varietal backgrounds and showed an average reduction in plant height of 50% combined with a significant increase in spikelet and ear fertility (but with a 22% reduction on grain size, depending on the varietal background). *Rht12,* however, showed a pleiotropic delay in ear emergence time as a result of its association with the awn inhibitor *B1* and therefore combines the reduction in height with an extended life cycle (Worland *et al*., 1994).

### 1.5.3 Molecular markers that are linked to the dwarfing genes

A study conducted by Cadalen *et al*. (1998) found the RFLP markers *Xfba1*-4B (4BS) and *Xfba211*-4D (4DS) to be linked to *Rht-B1* and *Rht-D1*, respectively. Ellis *et al*. (2002) developed PCR-based markers specifically for *Rht-B1b* and *Rht-D1b* which are able to distinguish between the dwarfing alleles and their corresponding tall alleles. Börner *et al*. (1997) found *Rht-B1c* to be distally linked to the two RFLP markers, *Xpsr144* and *Xpsr584* which are located near the centromere on chromosome arm 4BS.

*Rht8* was found to be tightly linked to the microsatellite marker *Xgwm261* on chromosome arm 2DS (Korzun *et al*., 1998). This was confirmed by Ellis *et al*. (2005) who identified an additional microsatellite marker, *Xwmc503*, linked to *Rht8* on chromosome arm 2DS. The micosatellite marker *Xgwm261* co-segregates with *Rht8* and produced three different allelic variants of 192 bp, 174 bp and 165 bp (Korzun *et al*., 1998). The diagnostic $Xgwm261_{192}$ allele was found in the source variety of *Rht8*, ‘Mara’, whereas the $Xgwm261_{165}$ and $Xgwm261_{174}$ alleles occurred in the varieties

'Saitama 27' and 'Norin 10', respectively (Worland *et al*., 1998a). However, it was subsequently found that presence of the *Xgwm261*$_{192}$ allele did not provide conclusive proof of the presence of *Rht8* and that 'Norin 10' derived varieties produced a haplotype where the *Xgwm261*$_{192}$ allele is not associated with reduced plant height (Ellis *et al*., 2007). Worldwide, approximately 90% of varieties have the 192 bp, 174 bp and 165 bp alleles with 6% of European varieties containing a 197 bp allelic variant. The remaining (4%) varieties carry allelic variants of between 195 bp and 215 bp (Worland *et al*., 2001). The 192 bp allele occurs particularly in Southern European wheat varieties but is also present in Chinese (60%) and Japanese (68%) wheat varieties. In north-western Europe and the UK the majority of cultivated varieties have the 174 bp allele whereas the 165 bp allele is common in CIMMYT derived varieties and in the countries using those varieties (Worland *et al*., 1998a; Worland *et al*., 2001). Ganeva *et al*. (2005) studied the allelic variation of *Xwms261* in 89 winter wheat varieties released in Bulgaria (76 modern, 13 old) and found that 84% of the modern varieties contained the 192 bp allele whereas only seven varieties had the 174 bp allele. A similar experiment was performed by Ahmad and Sorrells (2002) when they screened 71 wheat varieties from 13 countries. They found that the majority of cultivars (78.76%) contained the 174 bp or 165 bp alleles with only 5.63% of the cultivars containing the 192 bp allele.

Ellis *et al*. (2005) mapped several dwarfing alleles with the aid of microsatellite markers by screening doubled haploid lines and recombinant inbred lines (RILs), segregating for GA sensitive dwarfing alleles. They found the microsatellite markers *Xwmc317* (2BL), *Xbarc102* (3BS), *Xbarc151* (5AL) and *Xwms577* (7BL) to be closely linked with the dwarfing alleles *Rht4*, *Rht5*, *Rht9* and *Rht13*, respectively. Börner *et al*. (1997) reported that the RFLP markers *Xwmg634* and *Xpsr921*, on chromosome arm 4DS, are closely linked with, and located proximally to *Rht-D1c*. *Rht12* was mapped on the long arm of chromosome 5A at the same locus, or very tightly linked to, the awn inhibitor gene (*B1)* and *β-Amy-A1,* on a chromosome fragment ancestrally translocated from chromosome 4A (Worland *et al*., 1994). Korzun *et al*. (1997) obtained similar results when they found *Rht12* to be located distally and linked to microsatellite marker, *Xgwm179*, on a segment of chromosome arm 5AL homoeologous to *Triticeae* chromosome 4L. Ellis *et al*. (2005) also found the microsatellite marker *Xwmc410* to be closely associated with *Rht12*.

## 1.6 Genetic regulation of flowering time

In wheat the time of flowering is a complex trait and the duration of the life cycle is influenced by vernalization genes (*Vrn*), photoperiod genes (*Ppd*) and earliness *per se* genes (*Eps*). The *Eps* genes are the only genes that are not affected by environmental changes. Earlier studies suggested that flowering time in hexaploid wheat is controlled by genes located on virtually all of the 21 chromosome pairs (Worland, 1996). Tailoring of the flowering time of new varieties to environmental conditions is essential to maximize yield potential. The natural variation that exists for photoperiod response, vernalization response and developmental rate between genotypes of different geographical origin (Hoogendoorn, 1985) allows for considerable manipulation of flowering time.

### 1.6.1 Photoperiod response

Photoperiod response plays an important role in the climatic adaptability of wheat varieties to environmental conditions and varieties may be classified as photoperiod-insensitive or photoperiod-sensitive. Photoperiod response genes are sensitive to day length and photoperiod sensitive genotypes require a day length of more than 12 hours before initiation and development of floral primordia occur (Worland, 1996; Butterworth and Worland, 1998). In photoperiod insensitive genotypes on the other hand, the time to ear emergence and flowering is not affected by day length. Although wheat and barley have both been classified as long day plants, their responses to variation in day length are different. The day length insensitive gene *Ppd-H1* in barley results in earlier flowering when exposed to long days whereas day length insensitive genes in wheat promote early flowering when exposed to a short day length (Laurie, 1997).

Welsh *et al*. (1973) identified two genes located on chromosomes 2D and 2B, respectively, that are responsible for the control of photoperiod insensitivity and designated them *Ppd-D1* (formerly *Ppd1*) and *Ppd-B1* (formerly *Ppd2*), respectively. Using chromosome substitution lines, Law et al. (1978) assigned a third photoperiod insensitive gene, *Ppd-A1* (formerly *Ppd3*) to chromosome 2A. Scarth and Law (1983)

found *Ppd-D1* to be located on the short arm of chromosome 2D, whereas Mohler *et al*. (2004) genetically and physically mapped *Ppd-B1* to the short arm of chromosome 2B.

The primary effect of *Ppd-1* genes is to stimulate the early development of floral primordial tissue, following suitable vernalization, thereby shortening the period to flowering (Börner *et al*., 1993). The effect of these genes is influenced by environmental conditions and in field trials (UK) *Ppd-D1* reduced the time to ear emergence by approximately eight days, *Ppd-A1* by five days and *Ppd-B1* by three days. In France these genes had a bigger effect in reducing the time to ear emergence with *Ppd-D1*, *Ppd-A1* and *Ppd-B1* accelerating flowering by 17 days, nine days and three days, respectively (Butterworth *et al*., 2001). Field trials with varietal substitution lines (*Ppd-D1a* insensitive allele) in the UK showed an average acceleration in flowering of three days (Butterworth and Worland, 1998).

Secondary pleiotropic effects of *Ppd-1* genes are the result of a shortened life cycle. In varieties containing *Ppd-D1,* the visible pleiotropic effects include reduced plant height, a reduction in number of fertile tillers and a reduction in number of spikelets developing per spike. It was also found that *Ppd-D1* may decrease the risk of frost damage in UK winter wheat by shortening the thermal duration between crop emergence and the beginning of stem elongation (Foulkes *et al*., 2004). The reduction in spikelet number is compensated for by an increase in grain set in the remaining spikelets due to increased fertility of primary and secondary florets. The conditions under which varieties containing *Ppd-D1* are cultivated (northern and southern Europe) usually have an influence on the intensity of the pleiotropic effects and whether a yield increase is obtained. The photoperiod insensitive gene, *Ppd-D1*, is the only photoperiod insensitive gene believed to occur in cultivated European varieties and was derived from the ancient Japanese variety ‘Akakomugi’. The hot and dry summers in Southern Europe require earlier flowering genotypes whereas the cooler summer temperatures and abundant summer rain associated with Northern Europe favors later flowering genotypes (Snape *et al*., 2001).

By stimulating early flowering, thereby avoiding summer stress and allowing complete grain filling, the *Ppd-D1* gene is associated with a yield advantage of 30%

in Southern Europe compared to a yield increase of 15% in Middle Europe (Worland, 1996). In two separate studies in Germany, *Ppd-D1* resulted in average yield increases of 7.7% and 9%, respectively, whereas in the UK a yield reduction of 1.8% with an annual variation of +9% to -16% was observed during a 10 year trial period (Börner *et al*., 1993; Worland *et al*., 1998b). The evaluation of six varietal *Ppd-D1a* substitution lines in the UK over five seasons showed a significant reduction in yield for all varieties which varied between 44.5% and 9.5% (Butterworth and Worland, 1998). In the UK, genotypes containing *Ppd-D1a* have a yield advantage in warmer dryer summer conditions, whereas cool damp summer conditions favor genotypes containing *Ppd-D1b* (Worland, 1996). Worland *et al*. (1998b) argued that *Ppd-A1* and *Ppd-B1*, which are less insensitive than *Ppd-D1*, may favor the cooler conditions in the UK and Germany. They evaluated lines containing *Ppd-B1* or *Ppd-D1* in replicated field trials over a three year period in mid-Germany and the UK and found that *Ppd-B1* lines had 7% higher yield than *Ppd-D1* in the UK whereas both *Ppd-D1* and *Ppd-B1* caused a yield reduction in Germany over the trial period.

## 1.7 Wheat rusts

The wheat rusts (stem rust, leaf rust and yellow rust) are the most important diseases of wheat and can cause serious economic losses. The wheat rusts are caused by fungi that belong to the genus *Puccinia* of the family Pucciniaceae of the order Uredinales of the class Basidiomycetes (Knott, 1989).

### 1.7.1 Wheat leaf rust

Leaf rust, also known as brown rust, is caused by *Puccinia triticina*, which is a pathogen of wheat, closely related species and triticale. Leaf rust symptoms are largely restricted to the leaf blades, although the leaf sheaths and glumes may also be infected under severe infections or in very susceptible cultivars (Knott, 1989; Roelfs *et al*., 1992). Leaf rust preferentially occurs in mild climatic conditions (10-30°C) and is characterized by orange-brown pustules that are randomly distributed on leaves (Murray *et al*., 1998). Leaf rust causes premature defoliation which results in the shriveling of seeds and therefore a reduction in yield (Knott, 1989). The shriveling of

seeds is aggravated under severe infection and moisture stress conditions. Yield loss by leaf rust is primarily the result of reduced floret set and is usually comparatively small (<10%) but may increase to more than 30% under severe conditions (Roelfs *et al*., 1992).

#### 1.7.1.1 Life cycle of leaf rust

Leaf rust has five spore stages (microcyclic) and has both sexual and asexual stages (heteroecious). Wheat and related grasses are the hosts for the asexual stages whereas the sexual stages occur on an alternate host in the Ranunculaceae and Boraginaceae families which includes species of *Thalictrum*, *Anchusa*, *Clematis* and *Isopyrum*. The sexual stage plays an important role in the generation of new virulence combinations through the recombination of virulence and avirulence factors as well as other genetic factors (Roelfs *et al*. 1992).

During the cyclic, epidemic stage on the host, *P. triticina* reproduces through urediospores that are produced by uredia. Urediospores are distributed by wind to nearby fields where it germinates on the leaves in the presence of free water. The germinating urediospores penetrate the leaf through stomatal openings from where it spread to host cells where it forms specialized feeding structures called haustoria. Under optimal conditions a period of 7-10 days is required to progress from spore germination to sporulation, but this may take longer at lower temperatures.

At the end of the growing season or under unfavorable conditions the production of uridiospores stops and teliospores are formed under the epidermis. In the new season the teliospores germinate, and undergo meiosis to produce basidiospores which are released under humid conditions to infect the alternate host. Being released under humid conditions, hyaline and sensitive to light, basidiospores can not spread over a wide area. The infection of the host results in the development of pycnia (two mating types, + and -) on the upper leaf surface; the production of pycniospores and the transfer of pycniospores between opposite mating types by insects, rain or cohesion. Shortly thereafter (7-10 days) aecia appears on the underside of the leaf on a host plant discharging aeciospores that are distributed by wind to wheat fields in close

proximity. The germination of the aeciospores on wheat gives rise to the formation of uredia, thereby completing the life cycle (Knott, 1989; Roelfs *et al*., 1992).

### 1.7.2 Wheat yellow rust

Wheat yellow rust, also known as stripe rust, is caused by *Puccinia striiformis* f.sp. *tritici*. Unlike leaf rust this pathogen requires cool temperatures to reproduce (Knott, 1989; Roelfs *et al*., 1992). Infection with *P. striiformis* results in long yellow-orange stripes of rust pustules on the leaves but other parts of the plant may also be affected (Murray *et al*., 1998). Yellow rust is the only wheat rust where a single infection results in many uredia. Yellow rust is responsible for early defoliation that causes the shriveling of kernels; however, the reduction in yield is much higher compared to leaf rust with losses of up to 75% (Knott, 1989).

#### 1.7.2.1 Life cycle of yellow rust

Only the asexual stage of yellow rust has been studied since an alternate host has not been discovered. Yellow rust has three spore stages, urediospores, teliospores and basidiospores. Since no alternate host has been identified the life cycle appears to consist of repeated cycles of the asexual uredial stage (Knott, 1989).

### 1.7.3 Rust control

Several control strategies were developed to combat wheat rusts. Firstly, susceptible volunteer plants and alternate hosts may be systematically eradicated. This may reduce rust infections, especially in areas where wheat is cultivated during the winter and over-summering is a sensitive stage in the life cycle of the rust. A delay in the planting of winter wheat may prevent early infection of the new crops while excessive nitrogen fertilization may also reduce infestations. The use of fungicides (active ingredients includes azoles and morpholines) may also be considered an alternative in the control of wheat rusts, but is only feasible where high yields are obtained, for example in Europe. Fungicides are both expensive and potentially harmful to humans and nature. The breeding of rust resistant cultivars is the most effective solution for the sufficient control of wheat rusts. The development of new rust resistant cultivars is

an ongoing process as new virulent races, that overcome existing plant rust resistance genes, continuously evolve (Knott, 1989; Roelfs *et al*., 1992; Murray *et al*., 1998).

## 1.8 Wheat rust resistance genes

Plant pathogens may be categorized as those that kill and colonize the host plant (nectrotrophs) or those that parasitize on the living host tissue (biotrophs) (Ayliffe and Lagudah, 2004). In the case of biotrophic rust fungi that are host specific, a compatible or incompatible interaction will occur between the pathogen and the host plant. The type of interaction depends on the presence or absence of avirulence (*Avr*) genes in the pathogen that coincide with resistance genes in the host plant (Eckardt, 2006). The response to infection is determined by the ability of a corresponding host plant receptor produced by a resistance gene to bind a particular molecular signal, produced by the invading pathogen. If the host plant carries a resistance gene that can sense the infecting pathogen, it will trigger a defense (hypersensitive) reaction. Flor (1971) proposed the gene-for-gene concept to explain the underlying mechanism whereby resistance genes in plants are only effective when a particular resistance (R) gene recognize a particular virulence gene in the pathogen. Host plant resistance genes can either be classified as race-specific (vertical resistance), where the host plant is resistant against particular pathogen races, or race non-specific (horizontal resistance) where the host plant is resistant to all races of a pathogen species (Prell and Day, 2001). Race-specific resistance is normally based on the triggering of a hypersensitive response reaction at the infection site. Some race-specific genes are effective at all stages of plant development whereas certain other genes are expressed only in mature plant tissues. Slow rusting or partial resistance genes are normally expressed in the adult plant stage; are conditioned by more than one gene; do not trigger a hypersensitive response and provide durable resistance against a broad range of pathogen races (Kolmer, 1996; Ayliffe *et al*., 2008).

The majority of cereal resistance genes codes for proteins that contain a central region with a nucleotide binding site (NBS), that can bind ATP or GTP, and a carboxy terminal region with degenerate leucine rich repeats (LRR). The terminal region of the intercellular NBS-LRR proteins can either consist of a region

homologous to the Toll and Interleukin-I (TIR) protein region or contain a coiled-coil (CC) motif (Ayliffe and Lagudah, 2004). Other less ordinary proteins produced by resistance genes include the serine/threonine kinases and extracellular LRR (eLRR) proteins. The latter consists of a single transmembrane region and either a kinase region or a short intracellular C end (Hammond-Kosack and Parker, 2003). Feuillet *et al*. (1997) identified a gene, preliminary named *Lrk10*, that is linked to the *Lr10* leaf rust resistance locus. The *Lrk10* gene encodes a receptor-like kinase that contains a new type of extracellular recognition domain not previously identified in other receptor-like kinases.

When an invading pathogen is recognized by the host plant, a series of events are triggered. Cytological alteration of the infected and neighboring cells is the first visible sign of the resistance response during which the cell nucleus and cytoplasm move towards the site of infiltration. In addition the cell membrane shows increased permeability and depolarization with $K^+$ ions leaking, and $H^+$ ions moving into the plant cells. The defense response also triggers an oxidative burst during which reactive oxygen species are produced in the form of $O_2^-$, $HO_2$, $H_2O_2$ and OH radicals (Prell and Day, 2001). The increased levels of $H_2O_2$ produced in the defense response result in apoptosis of infected areas and activate the transcription of genes encoding enzymes associated with cellular protection, for example S-transferase and glutathione peroxidase (Levine *at al*., 1994). The resistance response may also stimulate lignification of the infected area as revealed when wheat was exposed to a *Puccinia graminis* f. sp. *tritici* elicitor (Kogel *et al*., 1991).

The use of resistant cultivars is the most effective way, environmentally and financially, to control rust infections in wheat. However, the use of single race-specific resistance genes (R) exerts strong selection pressure on the pathogen that will soon result in the selection of pathogen mutations that can evade recognition, thus rendering the genes ineffective. New breeding strategies are increasingly being used by breeders to produce varieties with more durable resistance through gene pyramiding. Varieties having several effective hypersensitive response resistance genes should provide longer-lasting resistance as the pathogen will need to mutate simultaneously at various effector loci in order to acquire virulence. The use of slow rusting, race non-specific genes in combination with race-specific genes can be

expected to provide even more durable resistance. However, rust pathogens with virulence to these resistance genes will eventually appear and continuous research to deploy new rust resistance genes is essential for effective crop protection against rust infections (Kolmer, 1996; Ayliffe *et al*., 2008).

## 1.9 Study aims

The foreign chromatin in the S14 translocation was derived from either the U or S genome of *Ae. kotschyi*. Apparently the complete 2DL chromosome arm had been replaced with *Ae. kotschyi* derived chromatin and as a result the translocated region does not pair with the normal wheat 2DL chromosome arm during meiosis (Marais *et al*., 2005). It is therefore not possible to map the translocation using conventional recombination based methods or to reduce the size of the translocation through conventional cross-breeding. Inheritance of the complete translocated chromosome arm as a single, large linkage block restricts the potential commercial utility of the *Lr54/Yr37* resistance. Firstly, the unnecessary species derived genes may have detrimental effects on yield and adaptation. While the complete translocation has not been tested extensively for associated detrimental effects, it would be of value to separate the resistance genes from possible height reducing and/or photoperiod response genes that may occur on the translocation. When combined with *Rht-B1b* (*Rht1*), which commonly occurs in South African wheats, the height reducing gene could result in dwarfed plants that are poorly adapted to the low yield, rain-fed conditions under which wheat is grown in South Africa. Thus, being able to also combine *Lr54/Yr37* with height reducing genes other than the gene carried on the translocation would be an advantage. Secondly, use of the complete translocation will rule out the possibility of combining *Lr54/Yr37* with useful wheat genes that occur on 2DL. The aim of the present study was therefore to genetically map the translocation and to attempt to obtain recombinant forms of the translocation that retained the resistance yet exchanged some of the unneeded *Ae. kotschyi* chromatin for homoeologous wheat chromatin. For this purpose a homoeologous pairing induction experiment was initiated by Marais *et al*. (2005; personal communication). Plants were produced that were heterozygous for the translocation yet lacked the *Ph1* locus and were therefore genotypically *ph1bph1b*. In the absence of *Ph1*, a low level of

homoeologous pairing of wheat and *Ae. kotschyi* chromatin was possible and could have produced hybrid chromosome 2DL arms. These plants were therefore testcrossed with W84-17. The testcross (04M144) $F_1$ were analyzed here in an attempt to identify and recover recombinants that could be used for genetic mapping.

The specific aims of this study were the following: (1) To initially identify plants with recombined chromosome 2DL arms by employing the most proximal and the most distal mapped wheat marker loci to screen the testcross population. (2) To further type the putative translocation recombinants obtained making use of DNA markers specific for chromosome arm 2DL. (3) Marais *et al*. (2005) did not provide conclusive proof of the presence of photoperiod insensitivity and height reducing genes on the translocation. It was necessary to conduct appropriate studies to confirm their presence/absence. If one of the genes, or both, occurred on the translocation it would have been necessary to screen the recombinants for their presence as well. (4) It was necessary to also screen the recombinants for the presence of both resistance genes so as to confirm that both were retained in the shortest recombinant. (5) Lastly, an attempt was made to find a DNA marker associated with the shortest recombinant that could be employed for marker assisted selection of the resistance.

# Chapter 2

# MATERIAL AND METHODS

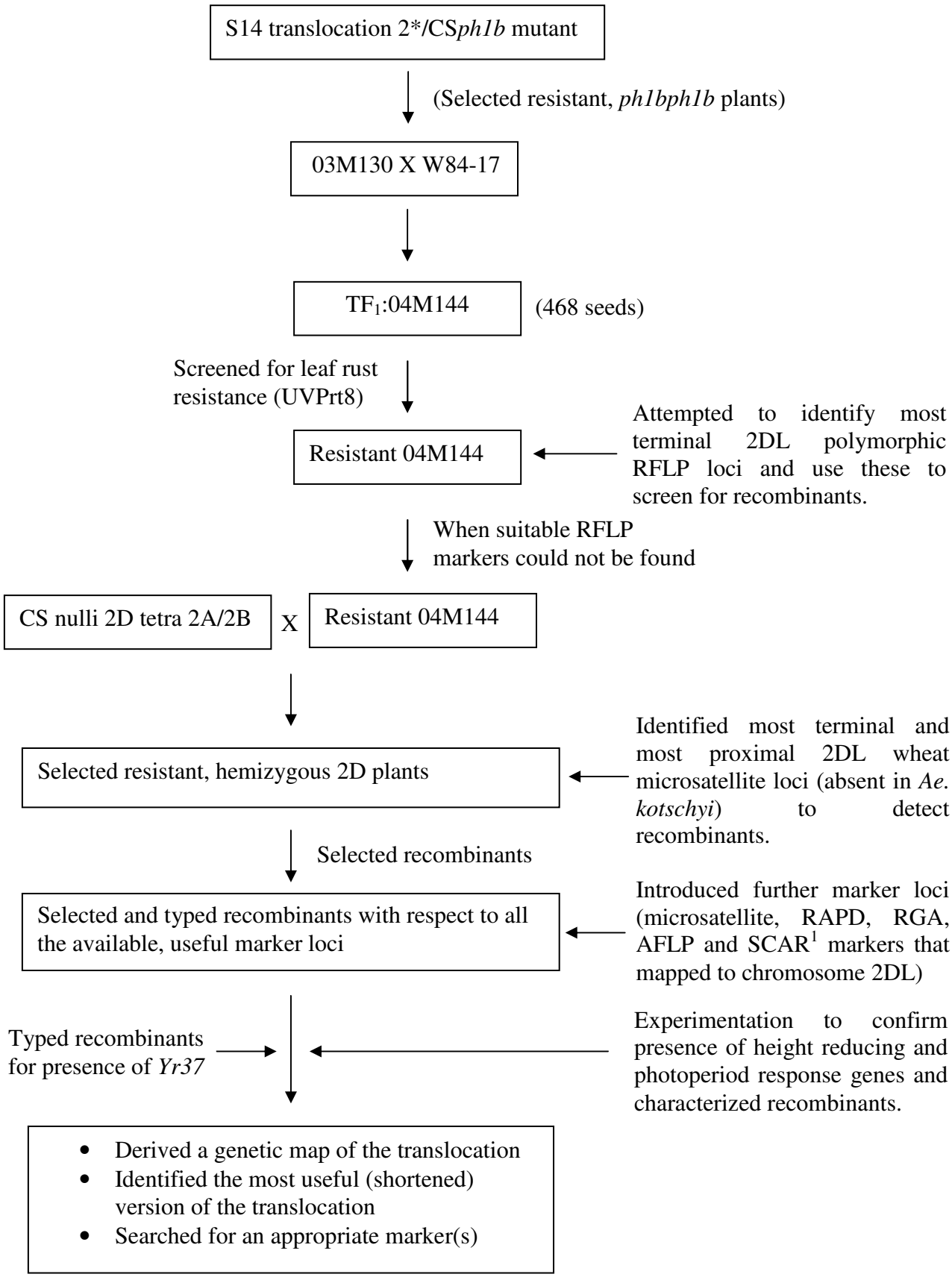
## 2.1 Study aims and outlines of experimentation

The primary aim of this study was to genetically map the major genes on the S14 translocation and at the same time use the information obtained to select the shortest, potentially most useful translocations that have retained the leaf and stripe rust resistance genes. A particular aim was to confirm the presence of photoperiod insensitivity and height reducing genes on the translocation, and if they existed, to find recombinants in which they were separated from the resistance genes.

The first part of this chapter outlines experimentation to identify and characterize possible allosyndetic recombinants for the translocation. This involved attempts to select a subset of putative recombinants and was followed by a search for further markers to confirm and characterize these. The second part of this chapter is concerned with attempts to verify the presence of *Rht* and *Ppd* loci on the S14 translocation, co-transferred with the resistance.

### 2.1.1 The identification of translocation recombinants

A broad outline of the experimental approach to produce, identify and characterize recombinant forms of the S14 translocation is presented in Fig. 2.1. Marais (2005; personal communication) produced cross 03M130, homozygous for the *ph1b* deletion and heterozygous for the S14 translocation. He test-crossed 03M130 plants with the leaf rust susceptible breeding line W84-17 and a total of 468 $TF_1$ (04M144) seeds were produced and provided for this study.

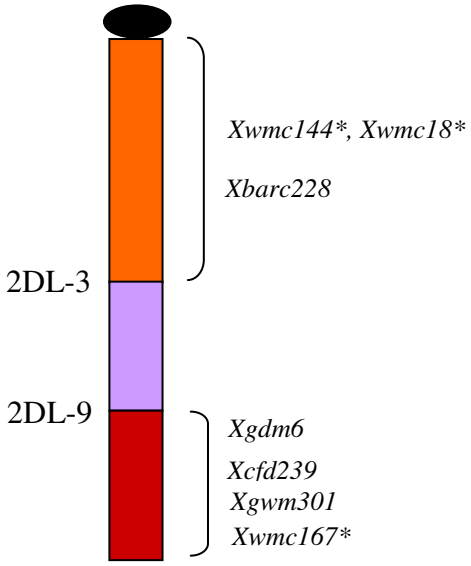



[1] Sequence characterized amplified regions

Figure 2.1 Outline of the strategy used to produce, recover and characterize recombinant forms of the CS-S14 translocation.

Nine RFLP loci, specific for chromosome arm 2DL (six with proximal and three with distal location) were tested for use in a first attempt to identify translocation recombinants. These markers were first hybridized to DNA of *Ae. kotschyi*, CS-S14 (heterozygous for the translocation), W84-17, CSN2AT2B, CSN2BT2A and CSN2DT2A (Table 2.1) to confirm their chromosome location and to determine if they detect polymorphic bands in the parents (*Ae. kotschyi*, CS and W84-17). When no useful RFLP polymorphisms could be found, it was decided to use wheat chromosome 2D microsatellite markers instead to identify recombinants. In order to apply the microsatellites (present in wheat but absent in *Ae. kotschyi* chromatin) it was necessary to first derive plants monosomic for the translocated chromosome 2D. To achieve this, each resistant $TF_1$:04M144 plant was used to pollinate CS nullisomic 2D tetrasomic 2A/2B plants. DNA extracts were made of the resistant progeny from these crosses and used for microsatellite analysis.

Seven microsatellite markers (Fig. 2.2) that map close to either the centromere or telomere of chromosome arm 2DL were initially identified from existing molecular and physical maps and tested on the genotype panel of Table 2.1 to select markers that are polymorphic and specific for chromosome arm 2DL. These were then used to identify translocation recombinants.




*The deletion bin location of these markers was deduced from their mapped locations on molecular maps of Somers *et al*. (2004) and Gupta *et al*. (2002).

Figure 2.2 Relative locations (Sourdille *et al*., 2004) within deletion bins of microsatellite loci for chromosome arm 2DL that were evaluated to find polymorphic markers that could be used to identify translocation recombinants.

Table 2.1 The genotype panel that was used to evaluate and select the microsatellite markers that could be used to screen for putative recombinants.

| No. | Genotype | Chromosome constitution |
|---|---|---|
| 1 | *Ae. kotschyi* accession 617 | 2n = 28, UUSS |
| 2 | W84-17 (common wheat breeding line) | 2n = 42, AABBDD |
| 3 | CS (common wheat breeding line) | 2n = 42, AABBDD |
| 4 | CSN2AT2B (CS nullisomic 2A tetrasomic 2B) | 2n = 42 |
| 5 | CSN2BT2A (CS nullisomic 2B tetrasomic 2A) | 2n = 42 |
| 6 | CSN2DT2A (CS nullisomic 2D tetrasomic 2A) | 2n = 42 |
| 7 | CSDT2DS[1] (CS ditelosomic 2DS) | $2n = 40 + 2t^{2DS}$ |
| 8 | CSDT2DL[2] (CS ditelosomic 2DL) | $2n = 40 + 2t^{2DL}$ |
| 9 | Monotelodisomic 2DS-$2D^{Transloc}$ (= CS-S14 hemi)[3] | $2n = 40 + t^{2DS} + 2D^{Transloc}$ |

[1] Derived from CS ditelosomic 2DS monotelosomic 2DL stock
[2] Derived from CS ditelosomic 2DL monotelosomic 2DS stock
[3] At the onset of the study an S14 translocation homozygote was not available. In order to provide a suitable translocation-carrying control a resistant $F_1$ was selected from the cross: CS ditelosomic 2DS monotelosomic 2DL (female parent)/CS-S14 translocation line.

### 2.1.2 Monosomic analysis

During the identification of putative translocation recombinants, a group of plants were encountered that produced questionable data. A second translocation to another chromosome in one of the 03M130 plants was thought to be the most plausible explanation. In order to investigate this possibility, a monosomic analysis was conducted to determine the chromosome location of the resistance in this group. For this purpose line 04M144-16 ($F_2$) was crossed with each of the 21 CS monosomics. Resistant $F_1$ monosomic plants were selected and selfed. $F_2$ progenies derived from the respective $F_1$ plants were then analyzed for seedling resistance to *P. triticina* pathotype UVPrt8. The deviant lines were also screened for the presence of *Lr19* using an STS (sequence tagged site) marker developed by Prins *et al*. (2001). Plants with *Lr19* produces a similarly strong infection type and were grown in the

greenhouse at the same time that the crosses to the CS nullisomic 2D plants were being made. Furthermore, monosomic analysis suggested the presence of a second rust resistance gene having the same chromosome location as *Lr19*.

### 2.1.3 Further characterization of the putative translocation recombinants

Additional chromosome 2D microsatellite markers were chosen from existing SSR-maps (Röder *et al*., 1998a; 1998b; Pestsova *et al*., 2000; Gupta *et al*., 2002; Guyomarc'h *et al*., 2002, Shi *et al*., 2003; Somers *et al*., 2004; Sourdille *et al*., 2004) and tested in an attempt to further characterize the translocation recombinants and to construct a genetic map of each. The additional chromosome arm 2DL loci included *Xgwm539*, *Xgwm157*, *Xcfd233*, *Xcfd50*, *Xgdm87*, *Xbarc1095* and *Xwmc41*. The chromosome 2DS loci were *Xgwm484*, *Xgwm261* (which is closely linked to the dwarfing gene *Rht8* – Korzun *et al*., 1998), *Xcfd116*; *Xcfd11* and *Xbarc124*.

In addition to the microsatellite markers, SCAR markers, Ust2-IIJ$_1^d$ and Sopw7, were mapped on the translocation. Marker Ust2-IIJ$_1^d$ was designed to label chromosome 2J$_1^d$ of *Thinopyrum distichum* (Marais *et al*., 2007) but also detects a locus on chromosome arm 2D of common wheat (Marais *et al*., 2009). Eksteen (2008) designed a SCAR marker, Sopw7, which amplifies similar sized fragments in several wild species derived translocations, including the S14 translocation (Marais 2008; personal communication.

In order to obtain still further markers that could be used to characterize the putative recombinants, AFLP methodology was employed for the detection of polymorphic fragments that map to the translocated segment.

### 2.1.4 Search for a unique *Ae. kotschyi*-specific marker associated with the shortest S14 translocation recombinant

When the shortest recombined translocation was identified, an attempt was made to also find a highly specific, dominant marker that could be used for its detection in marker-aided selection. Towards this end, RGA, RAPD and AFLP polymorphisms were generated and evaluated.

### 2.1.5 Possible presence of height reducing and photoperiod insensitivity loci on the S14 translocation

If such genes are located on the S14 translocation it may hamper the use of the resistance in some breeding programs and it was therefore necessary to confirm this. Should the genes occur on the translocation, it would furthermore be necessary to map them and attempt to also derive recombinants lacking them.

## 2.2 Experimental detail and protocols

### 2.2.1 Restriction Fragment Length Polymorphism (RFLP) analysis

Nine RFLP probes were used in an attempt to identify suitable markers that could be used to screen for translocation recombinants in the cross 04M144. The probes used were kindly provided by the following institutions: The PSR probes (De Vos *et al*., 1993) were isolated from genomic and cDNA libraries of wheat and were provided by Dr. P. Stephenson (John Innes Centre, Norwich Research Park, Colney, Norwich, NR4 7UH, UK). The FBA and FBB probes were supplied by Dr. M. Bernard (Institut National la Recherche Agronomique (INRA), Domaine de Crouelle – 234, avenue du Brézet – 63039 Clermont-Ferrand cedex 2, France) while the KSU probe (*Ae. tauschii*; Gill *et al*., 1991) was isolated from a genomic library and was supplied by Dr. K.S. Gill (Department of Plant Pathology, Kansas State University, Manhattan, Kansas 66506 USA).

All but one probe, KSUH16, were grown under ampicillin selection (100 µg/ml) while carbenicillin selection (50 µg/ml) was used for the remaining probe. The probes used and the vectors they were cloned into, together with the PCR primer sequences and selective antibiotic for each vector, are summarized in Table 2.2.

#### 2.2.1.1 Transformation of ultra-competent cells

The transformation of competent cells (*DH5α*) was performed using the protocol described in the Promega Protocols and Applications Guide (1991) – (2800 Woods Hollow Road, Madison, WI 53711-5399, USA) with small changes. One µg of dried

Table 2.2 A summary of the RFLP probes used and the vectors they were cloned into. The primer sequence and selective antibiotic for each vector are also given.

| Probe | Clone vector | Vector PCR primer sequence left | Vector PCR primer sequence right | Antibiotic selection |
|---|---|---|---|---|
| FBA074 | pBlueScript | AACAGCTATGACCATG | GTAAAACGACGGCCAGT | Ampicillin |
| FBB099 | pBlueScript | AACAGCTATGACCATG | GTAAAACGACGGCCAGT | Ampicillin |
| FBA209 | pBlueScript | AACAGCTATGACCATG | GTAAAACGACGGCCAGT | Ampicillin |
| PSR112 | pUC 18 | GTTTTCCCAGTCACGAC | CAGGAAACAGCTATGAC | Ampicillin |
| PSR151 | pUC 18 | GTTTTCCCAGTCACGAC | CAGGAAACAGCTATGAC | Ampicillin |
| PSR390 | pUC 18 | GTTTTCCCAGTCACGAC | CAGGAAACAGCTATGAC | Ampicillin |
| PSR571 | pUC 8 | GTTTTCCCAGTCACGAC | CAGGAAACAGCTATGAC | Ampicillin |
| PSR609 | pUC 8 | GTTTTCCCAGTCACGAC | CAGGAAACAGCTATGAC | Ampicillin |
| KSUH16 | pUC 18 | CACCTTCTTCTTCTTCTACT | ACACTCTTCATTGGCATCTC | Carbenicillin |

plasmid, containing the probe of interest, was dissolved in 5 µl of $dH_2O$ where after 1 µl (200 ng) of plasmid DNA was added to a 100 µl aliquot of competent cells thawed on ice. This cell suspension was mixed gently and incubated on ice for 20 min. The cells were placed in a 42°C water bath for 45-60 s and immediately quenched on ice for 2 min where after 900 µl of LB broth was added and the cells shaken gently (150 rpm) at 37°C for 90 min. Following incubation, 100 µl of the cell culture was plated out on LB agar plates containing the appropriate antibiotic. The remainder of the cell culture was centrifuged at 10,000 rpm for 30 sec and the supernatant discarded. The cells were re-suspended in the LB broth that remained (approx. 100 µl) after the supernatant had been discarded and were plated out on LB agar plates supplemented with the appropriate antibiotic. The LB agar plates with the cell culture were incubated at 37°C for 12-14 hours.

### 2.2.1.2 Alkaline lysis plasmid DNA isolation

Alkaline lysis plasmid DNA isolation was done as explained in the Promega Protocols and Applications Guide (1991) with small changes. Following cell transformation a single colony was selected and grown overnight in 5 ml of LB broth supplemented with the appropriate antibiotic (37°C, 225 rpm). In the case of a stab culture a sample was taken from the centre of the stab culture, inoculated in 5 ml of LB broth with the appropriate antibiotic and incubated overnight at 37°C while shaking (225 rpm). Following cell proliferation 1.5 ml of the overnight culture was transferred to a 1.5 ml microfuge tube and centrifuged at 12,000 rpm for 1½ min at room temperature. A freezer culture was made from the remainder of the overnight culture when 700 µl of the overnight culture was added to 300 µl of 50% (v/v) glycerol, mixed gently and stored at -80°C for future use. The medium was removed and the pellet re-suspended in 100 µl cold lysis buffer (50 mM Glucose, 25 mM Tris-Cl (pH 8.0) and 10 mM EDTA (pH 8.0)). This cell suspension was incubated at room temperature for 5 min after which 150 µl of a freshly prepared NaOH solution (0.2 M NaOH, 1% (w/v) SDS) was added, mixed by inversion and incubated on ice for 5 min. One hundred and fifty µl of a cold potassium acetate solution (3 M potassium, 5 M acetate, pH 4.8) was added and gently mixed by vortexing. This cell suspension was incubated on ice for 5 min and centrifuged at 12,000 rpm for 5 min at 4°C. The supernatant was transferred to a clean 1.5 ml microfuge tube, RNaseA added to a final concentration of 20 µg/ml and incubated at 37°C for one hour. One volume of phenol chloroform-isoamyl alcohol (25:24:1) was added, mixed by inversion and centrifuged at 12,000 rpm for 2 min. The aqueous upper phase was transferred to a clean 1.5 ml microfuge tube, extracted with one volume of chloroform-isoamyl alcohol (24:1) and centrifuged at 12,000 rpm for 2 min. The supernatant was carried over to a new 1.5 ml microfuge tube and the DNA precipitated with 2.5 volumes of cold 100% ethanol. Samples were incubated at 4°C for 30 min where after they were centrifuged at 12000 rpm for 15 min at 4°C. The supernatant was discarded and the pellet washed twice with 1 ml of cold 70% ethanol. The pellet was air dried and dissolved in 15 µl of $dH_2O$.

### 2.2.1.3 Probe labeling

PCR labeling of the plasmid insert (probe) was performed in 0.2 ml thin wall PCR tubes and consisted of a labeling reaction and a control. The labeling reaction contained 2 ng template DNA, 0.6 µM of the forward and reverse primer, 2 X dNTP labeling mix (Roche), 1 X PCR buffer, 1.5 mM $MgCl_2$, 2.5 units *Taq* DNA polymerase (Bioline) and SABAX water for a total reaction volume of 50 µl. The control reaction was performed in a 20 µl reaction volume. It contained 1 ng of template DNA, 0.5 µM of the forward and reverse primer, 0.2 mM dNTPs, 1 X PCR buffer, 1.5 mM $MgCl_2$, 1 unit *Taq* DNA polymerase (Bioline) and $dH_2O$. The RFLP probes were cloned into different plasmids which required a specific primer set to be used for each plasmid in order to amplify the inserted probe (Table 2.2). Amplification was performed in a thermal cycler (Applied Biosystems) as follows: one cycle at 94°C for 7 min, 36 cycles at 94°C for 45 s, 60°C for 1 min and 72°C for 2 min followed by a final extension of the amplified product at 72°C for 2 min and a soak temperature of 4°C. Two µl of the control reaction and 5 µl of the labeled reaction were loaded together with a molecular size marker on a 1.5% agarose gel with ethidium bromide (EtBr) and run in 1 X TBE in order to confirm that the labeling reaction was successful in which case the product of the labeled reaction should be larger than that of the control.

### 2.2.1.4 Restriction digestion of genomic DNA

Restriction digestion was performed in a 50 µl reaction volume which consisted of 10 µg of gDNA, 30 units of *Hind*III restriction endonuclease (Roche), 1 X restriction buffer and $dH_2O$. Another 10 units of restriction endonuclease were added the next morning and the reaction incubated for another 5 hours at 37°C where after the restriction digestion was terminated when 8.0 µl of loading dye (15% (w/v) Ficoll 400, 0.1% (w/v) Orange G, 0.06% (w/v) Bromophenol blue, 0.06% (w/v) Xylene cyanol FF and 30 mM EDTA (pH 8.0)) were added. The restriction fragments were size separated on an a 0.8% (w/v) agarose gel together with a DIG labeled molecular weight marker III (Roche) and run overnight at 30 V in 1 X TBE running buffer. Following gel electrophoresis the gel was stained with EtBr (1 µg/ml) and checked for product separation where after it was depurinated in 0.2 N HCl for 15 min. Following

depurination the gel was rinsed briefly with $dH_2O$ and transferred to a container with denaturing solution (1.5 M NaCl and 0.5 M NaOH) for 15 min. This treatment was repeated with fresh denaturing solution where after the gel was carried over to a container with neutralization solution (0.5 M Tris-Cl (pH 7.5) and 3 M NaCl) and washed twice for 15 min. The washing steps were performed at room temperature while shaking gently.

The DNA was transferred from the gel to a positively charged nylon membrane (Roche) according to the manufacturer's instructions using 20 X SSC (3 M NaCl and 0.3 M $Na_3$citrate, pH 7.0) as transfer buffer. The transfer process was allowed to proceed overnight where after the blot was removed and cross-linked under UV light (Spectroline Transilluminator) for 3 min. The blot was rinsed in a 2 X SSC solution, sealed in a plastic bag and stored at 4°C.

**2.2.1.5 Probe hybridization**

Twenty-five ml of hybridization buffer (*DIG Easy Hyb* - Roche) was preheated to 42°C in the roller of a hybridization oven (Techne hybridiser HB-1D). The blot was placed in the roller containing the hybridization buffer and pre-hybridized at 42°C for 3 hours if used for the first time and 90 min if used previously. Ten and a half µl of labeled probe was dissolved in 40 µl of $dH_2O$ and denatured in a boiling water bath for 5 min. The denatured probe was chilled on ice, added to a tube containing 25 ml of hybridization buffer and mixed by inversion. Following pre-hybridization the pre-hybridization buffer was drained and stored at -20°C. The hybridization buffer with the probe was added to the roller and the blot incubated overnight at 42°C.

Following hybridization the hybridization buffer was poured off (stored at -20°C) and the blot washed with post hybridization wash buffer 1 (2 X SSC and 0.1% SDS – high stringency) for 5 min at room temperature. This wash step was repeated with fresh wash buffer before the blot was returned to the roller and washed twice with pre-heated (68°) post-hybridization buffer 2 (0.5 X SSC and 0.1% SDS – low stringency) for 15 min at 68°C.

### 2.2.1.6 Chemiluminescent detection

The buffer solutions used for detection (*DIG Wash and Block Buffer Set* – Roche) were prepared according to the manufacturer's instructions. Following the post-hybridization washes the blot was rinsed briefly in 2 X SSC, transferred to a container with washing buffer and incubated for 1 min at room temperature. The wash buffer was replaced with buffer 2 (blocking buffer) and incubated for one hour at room temperature while shaking gently. *Anti-Digoxigenin-alkaline phosphatase* (Roche) was used for the detection of *DIG* labeled DNA. Two and half µl of *DIG* antibody was diluted in 50 ml of buffer 2 and mixed well. Following the blocking wash step, buffer 2 was drained and replaced with the freshly prepared diluted *DIG* antibody solution. The blot was incubated while shaking at room temperature for 30 min where after the antibody solution was discarded and the blot rinsed twice with washing buffer for 15 min while shaking at room temperature. *CDP star* (Roche) was used for the visualization of *alkaline phosphatase* linked to the *DIG* labeled DNA. Twenty µl of *CDP star* was added to 40 ml of buffer 3 (detection buffer) and mixed well. Following the washing buffer wash step, the blot was transferred to a container with buffer 3 and equilibrated at room temperature for 3 min where after buffer 3 was discarded and the blot rinsed in *CDP star* for 1 min. The blot was placed between two sheets of plastic and the edges sealed after all air bubbles were removed. The blot was placed in an X-ray cassette and exposed to X-ray film (Hyperfilm™ ECL – Amersham Biosciences) for 1-3 hours. Following exposure the blot was rinsed in $dH_2O$ and washed twice with strip solution (0.2 M NaOH and 0.1% SDS) at 37°C for 15 min after which the blot was rinsed with 2 X SSC for 5 min at room temperature, sealed and stored at 4°C.

### 2.2.2 Microsatellite analysis

In addition to the microsatellite loci listed in Fig. 2.2, 12 more microsatellite markers (given in section 2.1.3) were evaluated. Each marker was tested on a control panel (Table 2.1) and those that were polymorphic and specific for the intended chromosome arm (listed in Table 2.3) were used to characterize the putative translocation recombinants. The primer sequences of all the microsatellite markers used in this study were obtained from the Graingenes website

(http://www.graingenes.pw.usda.gov). The primer sequences together with their origin, annealing temperatures and map locations are summarized in Table 2.3.

#### 2.2.2.1 Microsatellite amplification and gel electrophoresis

Microsatellite amplification was performed in 20 µl reaction volumes using an Applied Biosystems thermal cycler. The reaction volumes contained 300 ng of template DNA, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer, 2 X PCR buffer, 1 unit of *Taq* DNA polymerase (Bioline) and $dH_2O$. The PCR cycling conditions consisted of one cycle at 94°C for 4 min whereafter 30 cycles were performed at 94°C for 30 sec, optimal annealing temperature for 30 sec and 65°C for 90 sec. A final extension step was performed at 65°C for 3 min and the product kept at a soak temperature of 4°C.

The PCR product was analyzed on a 1 mm thick 6% (w/v) denaturing poly-acrylamide (Acrylamide : Bis – 19:1) sequencing gel with 6 M urea and 1 X TBE. Eight hundred µl of 10% (w/v) ammonium persulphate and 160 µl of N,N,N',N'-Tetramethylethylendiamine (TEMED) were added to 160 ml of gel solution before casting the gel. The gel solution was mixed briefly and poured between two glass plates (39 cm x 33 cm / 41 cm x 33 cm) treated with y-Methacryloxypropyl-trimethoxysilane (Promega) and C-thru repelling agent, respectively. A comb was inserted and the gel was left for one hour to polymerize. Following gel preparation the gel was placed on an S 2001 sequencing apparatus (Life Technologies™) and pre-run at 60 W for 30 min in 1 X TBE running buffer. Prior to loading the samples an equal volume of AFLP loading buffer (98% (v/v) formamide, 10 mM EDTA (pH 8.0), 0.05% (w/v) bromophenol blue and 0.05% (w/v) xylene cyanol FF) was added to each sample, denatured at 95°C for 5 min and immediately placed on ice. Following the pre-run the wells were flushed with 1 X TBE and 12 µl of each sample was loaded together with a 100 bp molecular size marker (Promega). Gel electrophoresis was performed under cooling at 65 W for approx. seven hours after which the DNA was visualized by silver staining.

Table 2.3 A summary of the chromosome 2D microsatellite markers that were used for the identification and mapping of translocation recombinants.

| **Microsatellite marker** | **Forward primer sequence (5'-3')** | **Reverse primer sequence (5'-3')** | **Ann. Temp (°C)** | **Map locations** |
|---|---|---|---|---|
| *Xbarc228* (2DL) | CCCTCCTCTCTTTAGCCATCC | GCACGTACTATTCGCCTTCACTTA | 58 | Shi *et al*. (2003) |
| *Xwmc167* (2DL) | AGTGGTAATGAGGTGAAAGAAG | TCGGTCGTATATGCATGTAAAG | 51 | Gupta *et al*. (2002) |
| *Xgdm6* (2DL) | GATCAATCAAGCATGTGTGTGT | GGATGCCATGCCAAAGTATT | 55 | Pestsova *et al*. (2000) |
| *Xgwm539* (2DL) | CTGCTCTAAGATTCATGCAACC | GAGGCTTGTGCCCTCTGTAG | 60 | Röder *et al*. (1998b) |
| *Xcfd233* (2DL) | GAATTTTTGGTGGCCTGTGT | ATCACTGCACCGACTTTTGG | 60 | Sourdille *et al*. (2004)* |
| *Xgwm157* (2DL) | GTCGTCGCGGTAAGCTTG | GAGTGAACACACGAGGCTTG | 60 | Röder *et al*. (1998b) |
| *Xwmc41* (2DL) | TCCCTCTTCCAAGCGCGGATAG | GGAGGAAGATCTCCCGGAGCAG | 61 | Gupta *et al*. (2002) |
| *Xcfd50* (2DL) | TTCTGCAACATTTTGTCCCA | CGTATGATCCTAACGAGGGC | 60 | Guyomarc'h *et al*. (2002) |
| *Xcfd116* (2DS) | TTTGCCCATTACAACAAGCA | CAAGCAGCACCTCATGACAG | 60 | Sourdille *et al*. (2004)* |
| *Xgwm484* (2DS) | ACATCGCTCTTCACAAACCC | AGTTCCGGTCATGGCTAGG | 55 | Röder *et al*. (1998b) |
| *Xgwm261* (2DS, *Rht8*) | CTCCCTGTACGCCTAAGGC | CTCGCGCTACTAGCCATTG | 55 | Röder *et al*. (1998b) |
| *Xbarc124* (2DS) | TGCACCCCTTCCAAATCT | TGCGAGTCGTGTGGTTGT | 52 | Shi *et al*. (2003) |

*Constructed a physical deletion map that included this marker

### 2.2.2.2 Silver staining

Following product separation the gel was removed from the sequencing apparatus and the glass plates separated using a plastic spatula. The small plate containing the gel was placed in a tray with fixation solution (10% v/v ethanol, 5% acetic acid) and incubated at room temperature for 20 min while shaking gently. Following fixation the gel was rinsed twice with $dH_2O$ for 5 min after which it was stained in $AgNO_3$ (0.1% w/v) for 20 min and rinsed briefly with $dH_2O$ for 10 sec. The gel was transferred to a tray containing developing solution (0.375 M NaOH and 0.15% v/v Formaldehyde which were added just before use) and shaken gently at room temperature until the first bands appeared (approx. 5-10 min) where after the gel was rinsed with $dH_2O$, air dried and sealed between two sheets of plastic.

### 2.2.3 Detection of sequence specific markers

SCAR markers Ust2-$IIJ_1^d$ and Sopw7 was used to screen *Ae. kotschyi*, CS-S14 (hemi), CS, W84-17 and ten putative recombinants. *STSLr19*$_{130}$ was used to determine whether the resistance in certain testcross progenies could be *Lr19* rather than the S14 translocation. The primer sequences and annealing temperatures of the STS and SCAR markers are given in Table 2.4.

The PCR amplification conditions for the *Lr19* specific marker locus, *STSLr19*$_{130}$, and the SCAR marker loci, *Xust2-IIJ*$_1^d$ and *Xsopw7*, were as follows: 200 ng template DNA, 1 X PCR buffer, 2.0 mM $MgCl_2$, 0.1 mM of each dNTP, 0.5 µM of the forward and reverse primer, 1 unit of *Taq* DNA polymerase (Bioline) and $dH_2O$ to a final volume of 25 µl. PCR amplification was performed using a thermal cycler from Applied Biosystems which was programmed as follows: one cycle at 94°C for 4 min followed by 30 cycles at 94°C for 30 sec, 30 sec at primer annealing temperature, 72°C for 30 sec and a single elongation step at 72°C for 5 min where after the samples were kept at a holding temperature of 4°C. The PCR samples together with a molecular size marker were size separated on a 1.5% agarose gel with EtBr.

Table 2.4 Primer sequences and annealing temperatures of the sequence specific markers used in this study.

| Marker | Forward primer sequence (5'-3') | Reverse primer sequence (5'-3') | Annealing Temp. (°C) |
|---|---|---|---|
| *Xust2-IIJ$_1$*$^d$ | TGCTCAATGAAACAGGAGAC TG | TTGAAACTTCCCTCCTGCG | 58 |
| *STSLr19$_{130}$* | CATCCTTGGGGACCTC | CCAGCTCGCATACATCCA | 60 |
| *Xsopw7* | CAGGAGCATAGTCATACTTG GG | CTGGACGTCAACAATGGC | 60 |

### 2.2.4 Random Amplified Polymorphic DNAs (RAPDs)

RAPD markers were also evaluated in an attempt to identify markers that are polymorphic for the shortest translocation recombinant. Thirteen RAPD primer sets, 230 primers in total, were obtained from Operon biotechnologies (http://www.operon-biotech.com) and tested by Eksteen (2008) who identified 12 RAPD primers that were polymorphic for the CS-S14 translocation but had poor reproducibility. These 12 primers were tested on a control panel consisting of *Ae. kotschyi*, CS-S14 (hemi), rec. 74, CS and W84-17. The RAPD primers that were tested, their sequences and annealing temperatures are listed in Table 2.5.

The PCR reaction was performed in a total reaction volume of 25 µl which consisted of: 40-80 ng of gDNA, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of primer, 0.5 U of *Taq* DNA polymerase (Bioline) and $dH_2O$. PCR amplification was done in a thermal cycler (Applied Biosystems) according to the following program: 5 min at 94°C; 45 cycles each consisting of 1 min at 94°C, 1 min at the primer annealing temperature (Table 2.5) and 2 min at 72°C; a final extension cycle at 72°C for 2 min and a soak temperature of 4°C. The PCR product together with a molecular size marker was separated on a 2% agarose gel with EtBr and visualized under UV light.

Table 2.5 RAPD primers used in an attempt to identify markers that are linked to the shortest translocation recombinant.

| RAPD | Primer sequence | Annealing temp. (°C) |
|---|---|---|
| OPE19 | ACGGCGTATG | 36.8 |
| OPF16 | GGAGTACTGG | 30.5 |
| OPN8 | ACCTCAGCTC | 34.3 |
| OPN12 | CACAGACACC | 33 |
| OPN13 | AGCGTCACTC | 44.5 |
| OPN20 | GGTGCTCCGT | 41.1 |

| RAPD | Primer sequence | Annealing temp (°C) |
|---|---|---|
| OPP4 | GTGTCTCAGG | 31.8 |
| OPQ7 | CCCCGATGGT | 39.8 |
| OPR4 | CCCGTAGCAC | 38.1 |
| OPT5 | GGGTTTGGCA | 37 |
| OPU9 | CCACATCGGT | 41.1 |
| OPU11 | AGACCCAGAG | 32.8 |

### 2.2.5 Amplified Fragment Length Polymorphism (AFLP)

AFLP markers were used in a further attempt to find polymorphims that map on the shortest translocation recombinant. AFLP analysis was performed on *Ae. kotschyi*, CS-S14 (hemi), CS, W84-17 and the 10 recombinants according to a protocol adapted from Donini and Koebner (John Innes Centre). Restriction digestion of gDNA and ligation were performed in the same reaction volume which consisted of 300 ng of gDNA, 1 X One-Phor-All buffer (Pharmacia), 1 mM ATP, 1 µl of each of the *Eco*RI and *Mse*I adaptors per reaction (ABI), 5 U of each of the *Eco*RI (Roche) and *Mse*I (New England Biolabs) restriction enzymes, 0.1 µg/µl BSA (New England Biolabs), 1U of T4 DNA ligase and water for a final reaction volume of 20 µl. The reaction was mixed briefly and left overnight at room temperature. Following restriction digestion and the ligation of adaptors, 15 µl of each ligation reaction was diluted (1:9) by adding 135 µl of 1 X $TE_{0.1}$ (10 mM Tris-HCL, 0.1 mM $Na_2EDTA$, pH8.0) whereas the remaining 5 µl was tested on 1.5% agarose gel with EtBr to see if the DNA was digested completely. A light smear would indicate complete digestion.

The pre-amplification reaction consisted of 5.2 µl of diluted digested template DNA with adaptors, 1 X PCR buffer, 0.2 mM of each dNTP, 1.5 µl pre-selective

primer mix per reaction (ABI), 1.5 mM $MgCl_2$, 1U of *Taq* DNA polymerase (Bioline) and water for a final reaction volume of 20 µl. Pre-amplification was done using an Applied Biosystems thermal cycler programmed as follows: 72°C for 5 min, 30 cycles at 94°C for 30 sec, 56°C for 60 sec, 72°C for 60 sec and a single elongation step for 5 min at 72°C with a soak temperature of 4°C. Following pre-selective PCR amplification, 135 µl of 1 X $TE_{0.1}$ was added to 15 µl of each sample (1:9) whereas the remaining 5 µl was tested on a 1.5% agarose gel with EtBr to see if pre-amplification was successful.

For selective amplification, selective *Eco*RI primers, labeled with different fluorescent dyes (*Eco*RI – ACA (FAM – blue), *Eco*RI – AGG (JOE – green), *Eco*RI – AAC (NED – yellow)), were used with unlabeled selective *Mse*I primers (CAG, CTG, CAT, CTC, CTA). Selective amplification was performed in multiplex reaction mixtures where all three florescence labeled *Eco*RI selective primers were used with a single unlabeled *Mse*I selective primer. The selective amplification PCR reaction consisted of 2.5 µl of diluted pre-amplification DNA, 1 X PCR buffer, 0.2 mM of each dNTP, 0.5 µM of *Mse*I selective primer (ABI), 0.05 µM of each of the three labeled *Eco*RI selective primers (ABI), 1.5 mM $MgCl_2$, 0.25 U of *Taq* DNA polymerase (Bioline) and water for a final reaction volume of 10 µl. Amplification was done using an Applied Biosystems thermal cycler with the following program: 13 cycles at 94°C for 30 sec, annealing temperature for 30 sec and 72°C for 1 min. An annealing temperature of 65°C was used in the first cycle and was lowered with 0.7°C for every following cycle. This was followed by 23 cycles at 94°C for 30 sec, 56°C for 30 sec and 72°C for 60 sec with a soak temperature of 4°C. Fragment separation was conducted with an ABI sequencer at the Stellenbosch University Central Analytical Facility (http://www.academic.sun.ac.za/saf/) and fragment analysis was done using Genemapper® softwear IV.

### 2.2.6 Resistance Gene Analogs (RGAs)

When comparing the protein products of cloned resistance genes from diverse plant species, structural similarities were found which created opportunities to amplify and isolate similar sequences in other plant species through the use of degenerate primers based on these conserved motifs (Leister *et al*., 1996). In this thesis nine primers that

are based on the conserved amino acid motifs of resistance genes in different plant species (tomato, wheat and rice) were used. The primers were designed and tested by Shi *et al*. (2001) and Yan *et al*. (2003) for the detection of polymorphic markers that are linked to yellow rust resistance genes in wheat. The primers were used to screen *Ae. kotschyi*, CS-S14 (hemi), the shortest translocation recombinant (rec. 74), CS and W84-17 in an attempt to identify an RGA marker linked to the *Lr54* gene on the shortest translocation. The primers were used in combination with each other and PCR products were size separated on a 6% poly-acrylamide denaturing gel (Chen *et al*., 1998). The primers used, their sequence, the resistance gene and the conserved motifs on which they are based are summarized in Table 2.6.

Table 2.6 RGA primers used together with their sequence and the resistance gene expressing the protein on whose conserved motif the primer is based.

| No. | Primer | Primer sequence (5'-3') | Resistance Gene | Domain |
|---|---|---|---|---|
| 1 | Pto kin1IN | AAGTGGAACAAGGTTACG | *Pto* | Kinase |
| 2 | Pto kin2IN | GATGCACCACCAGGGGG | *Pto* | Kinase |
| 3 | Cre3LR-R | CAGGAGCCAAAAATACGTAAG | *Cre3* | LRR[1] |
| 4 | Xa1LR-F | CTCACTCTCCTGAGAAAATTAC | *Xa1* | LRR[1] |
| 5 | Xa1LR-R | GAGATTGCCAAGCAATTGC | *Xa1* | LRR[1] |
| 6 | Xa1NBS-F | GGCAATGGAGGGATAGG | *Xa1* | NBS[2] |
| 7 | Xa1NBS-R | CTCTGTATACGAGTTGTC | *Xa1* | NBS[2] |
| 8 | CLRR-INV1 | GCAGCAACTTGTGC | *Cf9* | LRR[1] |
| 9 | XLRR-INV2 | GAGGAAGGACAGGTTGCC | *Xa21* | LRR[1] |

[1] LRR – Leucine-rich repeat
[2] Nucleotide-binding site

### 2.2.6.1 RGA amplification and gel electrophoresis

Each PCR reaction consisted of 100 ng of template DNA, 1 X PCR buffer, 1.2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.32 µM of each primer, 1 U of *Taq* DNA polymerase (Bioline) and $dH_2O$ for a total PCR reaction volume of 25 µl. PCR amplification was

carried out in a thermal cycler (Applied Biosystems) which was programmed as follows: 5 min at 94°C followed by 45 cycles of 1 min at 94°C, 1 min at 45°C and 2 min at 72°C with a ramp time of 2½ min between the denaturing and annealing steps. Amplification was rounded off with a final extension step of 7 min at 72° and a soak temperature of 4°C. Fifteen µl of AFLP loading buffer was added to each PCR product and 15 µl of each sample together with a 100 bp molecular size marker were loaded on a 6% (w/v) denaturing poly-acrylamide sequencing gel. Gel electrophoresis and staining were done as described in sections 2.2.2.1 and 2.2.2.2.

### 2.2.7 Development of a dominant marker specific for the shortest recombinant

An attempt was made to convert an AFLP polymorphism specific for the shortest recombinant to a PCR based SCAR marker. AFLP analysis was initially performed using fluorescently labeled primers. In order to recover the polymorphic fragment, clone and sequence it, the amplification reaction had to be repeated using unlabeled primers. Following unlabeled selective amplification the product was size separated on a 6% (w/v) denaturing poly-acrylamide sequencing gel and silver stained. Since a large number of bands were visualized by silver staining, the polymorphic band was not visible as a separate entity. The region that contained the polymorphic band (as revealed by pherograms) was therefore excised from the *Ae. kotschyi* and CS (negative control) lanes and dried at room temperature. The DNA was eluted from the dried gel using 50 µl of 1 X $TE_{0.1}$ (10 mM Tris-HCL, 0.1 mM $Na_2EDTA$, pH 8.0) and the DNA precipitated using 1/10 volume of 3 M sodium acetate (pH 5.0) and 2 volumes of cold ethanol after which the DNA was dissolved in 15 µl of $dH_2O$. PCR amplification was performed using 5 µl of DNA, 1 X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM *Mse*I primer, 1 µM unlabeled *Eco*RI primer, 0.25 U *Taq* DNA polymerase (Bioline) and $dH_2O$ for a final reaction volume of 20 µl. The same PCR program as for selective amplification was used. The PCR product was size separated on a 2% agarose gel with EtBr, the fragment of interest excised and the DNA recovered using Sigma's GenElute gel extraction kit. The DNA was precipitated using 1/10 volume of 3 M sodium acetate (pH 5.0) and 2 volumes of cold ethanol and the DNA dissolved in 15 µl of $dH_2O$. The *Ae. kotschyi* fragments were cloned using the pGem – T Easy vector (Promega) according to the manufacturer's instructions and the clones were transformed into competent *DH5α* cells as described in section

2.2.1.1. The cells were plated out on LB agar plates containing ampicillin (100 µg/ml), 100 µl Isopropyl B-D –thiogalactoside (IPTG - 0.1 M) and 20 µl 5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside (X-gal - 20 mg/ml) and incubated at 37°C for 12-14 hours. White colonies and a number of blue colonies (control) were selected and suspended in 10 µl of $dH_2O$ after which they were screened using the T7 and Sp6 vector primers. PCR amplification was as follows: 3 µl of colony DNA, 1 X PCR buffer, 1.5 mM $MgCl_2$, 0.05 mM of each dNTP, 0.25 µM of each primer, 0.5 U of *Taq* DNA polymerase (Bioline) and $dH_2O$ for a final reaction volume of 10 µl. PCR amplification was conducted using a thermal cycler from Applied Biosystems that was programmed as follows: one cycle at 94°C for 5 min followed by 25 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. The program was rounded off with a final extension step at 72°C for 7 min and a soak temperature of 4°C. The PCR product together with a molecular size marker was separated on a 2% agarose gel with EtBr (1 x TBE) to identify those colonies containing the correct insert size. Following identification of colonies with the correct insert, the remainder of the colony suspension was used to inoculate 5 ml of LB broth containing ampicillin (100 µg/ml) and grown overnight at 37°C while shaking (225 rpm). Plasmid DNA isolation was conducted the following morning using Promega's DNA purification kit and the plasmid insert was sequenced at the Stellenbosch University Central Analytical Facility (http://www.academic.sun.ac.za/saf/). Sequence analysis was done using the program BioEdit and primers were designed using the program OligoAnalyzer.

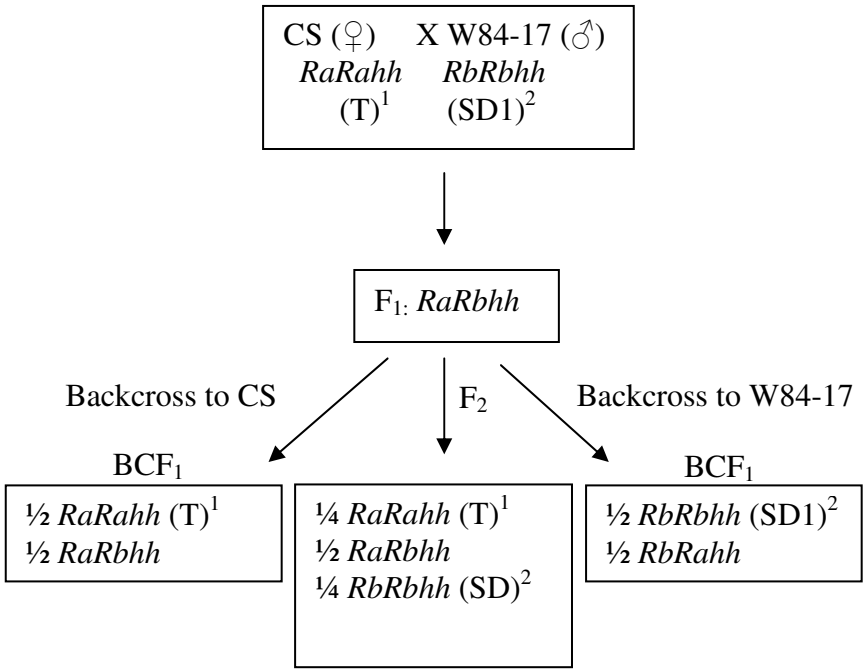
The designed primers were evaluated on a panel of resistant and susceptible $BCF_2$ and $BCF_3$ plants segregating for the shortest recombinant translocation as well as the 10 translocation recombinants. The PCR reaction mixture consisted of 200 ng of template DNA, 1 X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer, 1 U of *Taq* DNA polymerase and $dH_2O$ to a final volume of 25 µl. A thermal cycler (Applied Biosystems) was programmed as follows: 5 min at 94°C, 35 cycles consisting of 1 min at 94°C, 1 min at 61°C and 1 min at 72°C followed by a final extension step at 72°C for 7 min and a soak temperature of 4°C. Following PCR amplification the PCR product was size separated on a 1.5% agarose gel (EtBr, 1 X TBE) together with a molecular size marker.

## 2.3 Confirmation of a height reducing gene on the S14 translocation

Marais et al. (2005) suggested that a height reducing gene may occur on the S14 translocation. This gene could be homoeo-allelic to the *Rht8* gene, however, there is disagreement about the chromosome 2D arm location of *Rht8* (Komugi Catalogue of Gene Symbols – http://www.shigen.nig.ac.jp; Korzun *et al*., 1998; Ellis *et al*., 2005); therefore the *Ae. kotschyi* derived height reducing gene in CS-S14 is here designated as *H*. Firstly, the cultivar CS (*Rht-B1a Rht-B1a hh*) was crossed with W84-17 (*Rht-B1b Rht-B1b hh* – Fig. 2.3a). This served as a control cross to provide an indication of the variation in height resulting from the segregation of *Rht* genes in the CS and W84-17 backgrounds. Secondly, CS was crossed with resistant (UVPrt8) plants selected from the near-isogenic line $F_1$:0514 (CS-S14/4*W84-17 – *Rht-B1b Rht-B1b Hh* – Fig. 2.3b). The resistant $F_1$:0514 plants would have been homozygous for the dominant *Rht-B1b* allele in W84-17 yet heterozygous for *H* which occurs on the S14 translocation. The progeny from each cross were backcrossed to both parents. The progeny from the cross $F_1$:0514 X CS were screened, and leaf rust resistant plants were selected for backcrossing to the respective parents. The parents, $F_1$ and $F_2$ derived progeny together with the respective backcrosses in both sets of crosses were screened for rust resistance and resistant and susceptible plants were grown in a greenhouse. If the translocation does contain a height reducing gene, the backcross and $F_2$ progeny in Fig. 2.3b should include dwarfs. On the other hand the progeny from the control crosses in Fig. 2.3a should consist of tall and semi-dwarf plants only.

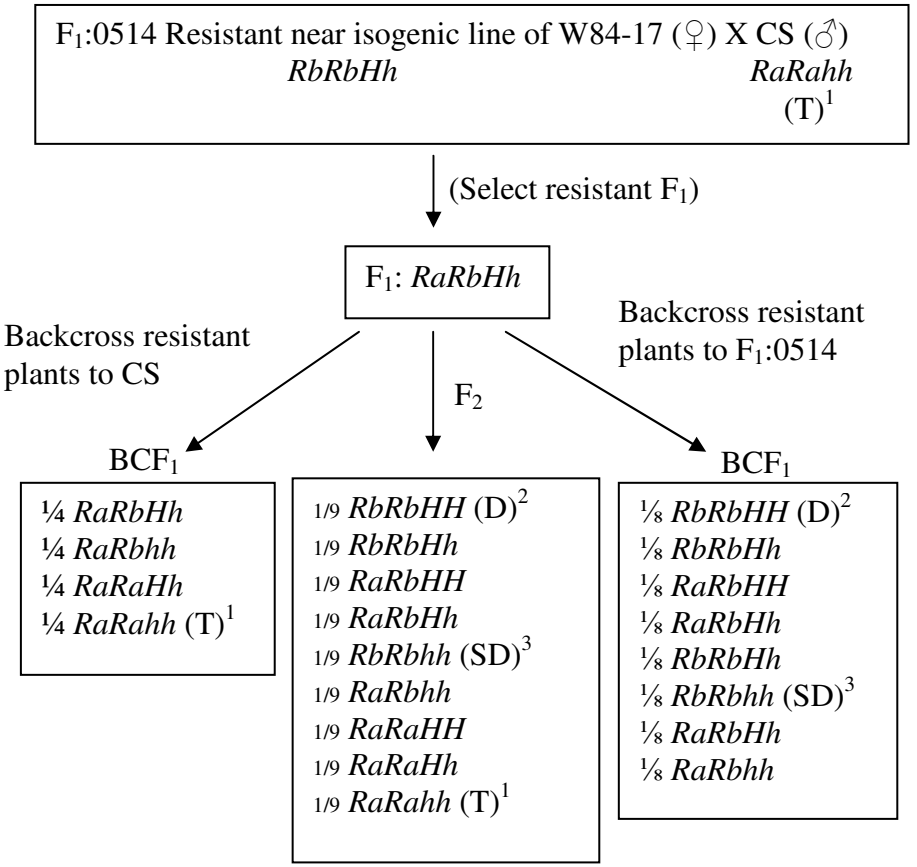
## 2.4 Characterization of S14 recombinants for the presence of *H*

Comparison of the range of plant height polymorphisms among the selfed progeny of a particular recombinant should reveal (if an ample sample is used) whether the height reducing gene, *H*, is still present. The procedure followed to obtain and screen the testcross $F_1$ 04M144 population for translocation recombinants is outlined in Fig. 2.4 and provides the possible plant height genotypes of the recombinants. Only the resistant testcross $F_1$ was used for further evaluation and they could potentially have had the genotypes *Rht-B1b Rht-B1b Hh* or *Rht-B1a Rht-B1b Hh* provided that the association between the resistance and H was not broken.




[1] T = tall phenotype; [2]SD = semidwarf phenotype associated with *Rht-B1b*

Figure 2.3a Outline of the control crosses made in an attempt to confirm the presence of a height reducing gene (*H*) on the S14 translocated segment. The meanings of genotypic symbols are as follows: *Ra* = *Rht-B1a*; *Rb* = *Rht-B1b*; *H* = height reducing gene that occurs on the S14 translocation; *h* = wheat 2DL chromatin region corresponding to the *H* locus.

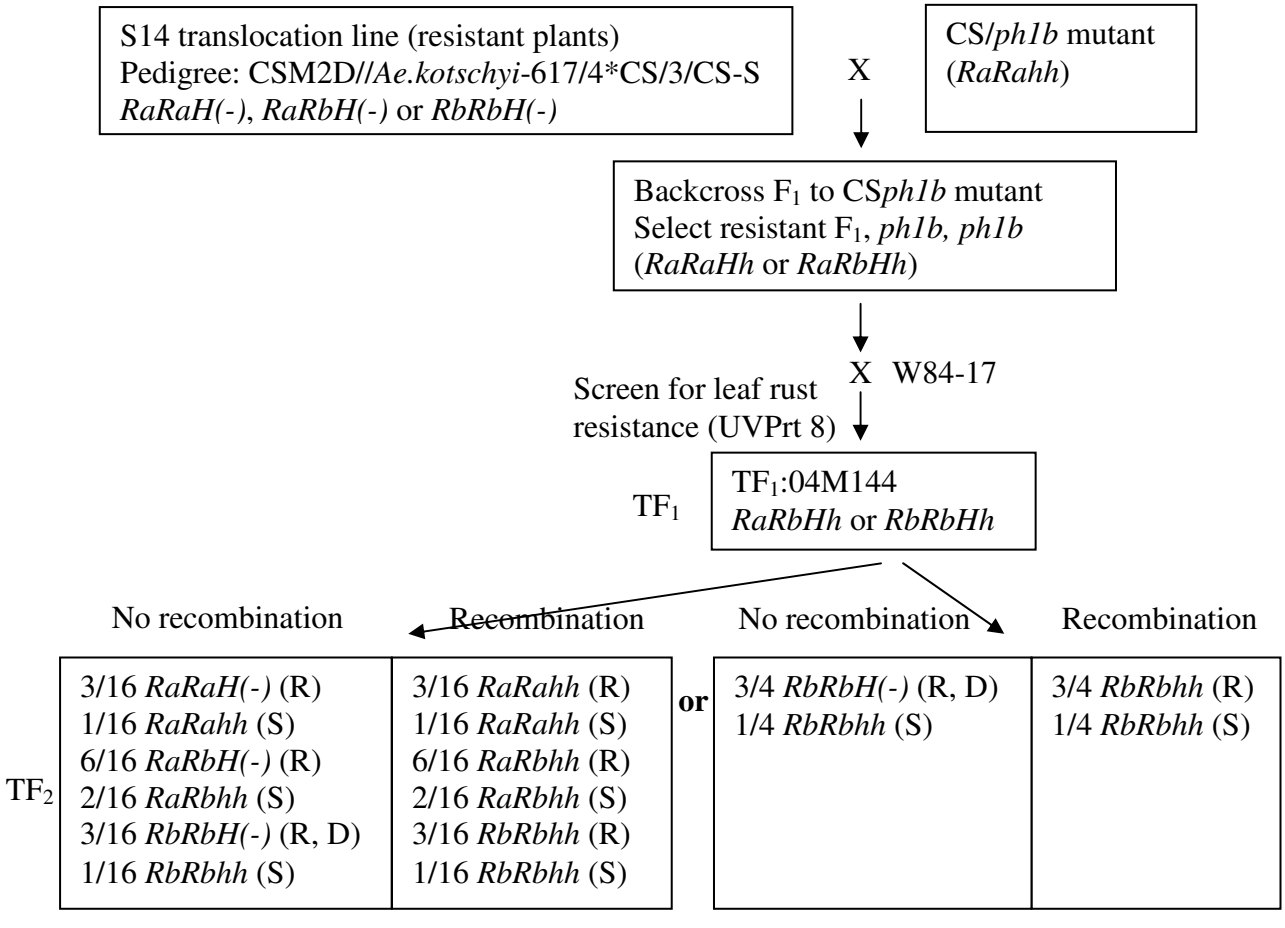



[1]T = tall phenotype; [2]D = dwarf phenotype; [3]SD = semi dwarf phenotype associated with the presence of *Rht-B1b*

Figure 2.3b Outline of the experimental crosses made in order to confirm the presence of a height reducing gene (*H*) on the S14 translocated fragment. The meanings of genotypic symbols are as follows: *Ra* = *Rht-B1a*; *Rb* = *Rht-B1b*; *H* = height reducing gene that occurs on the S14 translocation; *h* = wheat 2DL chromatin region corresponding to the *H* locus.

Thus, in the absence of homoeologous recombination between the two genes, the resistant $TF_2$ derived from the *Rht-B1b Rht-B1a Hh* resistant $TF_1$ would have included 18.75% resistant dwarfs *(Rht-B1b Rht-B1b H(-))* whereas the *Rht-B1b Rht-B1b Hh* progeny of the resistant $TF_1$ would have included 75% resistant dwarf plants. However, if *H* was lost through homoeologous recombination, that particular $F_2$ would not have included resistant dwarfs.

$F_2$ and $F_3$ progeny of each recombinant, together with controls CS, W84-17 and CS-S14 were evaluated for plant height in an attempt to determine whether the specific recombinant had retained *H*. Few (often only 2-3) $F_2$ seeds of each recombinant were available; therefore $F_3$ seeds segregating for the resistance were also grown in a field trial and were evaluated for plant height.




R = Resistant; S = Susceptible; D = Dwarf

Figure 2.4 Strategy for testing the putative recombinants for the presence of the height reducing locus, *H*. The meanings of genotypic symbols are as follows: *Ra* = *Rht-B1a*; *Rb* = *Rht-B1b*; *H* = height reducing gene that occurs on the S14 translocation; *h* = wheat 2DL chromatin region corresponding to the *H* locus.

Clearly, occurrence of dwarf plants confirmed the presence of *H*, however, absence of dwarfs among the progeny did not conclusively show that *H* was no longer present. This was due to segregation at the *Rht-B1* locus combined with the small numbers of $F_1$ and $F_2$ seeds that were sampled.

In an attempt to confirm the absence of *H* in the shortest recombinant, $BCF_3$ (CS-S14 translocation/2*CS*ph1b* mutant//W84-17/3/CSN2DT2A-B/4/2*W84-17) progeny together with control lines CS, CS-S, W84-17 and CS-S14 were evaluated for adult plant height in a greenhouse experiment. $BCF_3$ seedlings were screened for leaf rust resistance (UVprt8) after which 20 resistant and 11 susceptible seedlings were transplanted to the greenhouse.

## 2.5 Testing for the presence of a photoperiod insensitivity gene (*Ppd*) on the S14 translocation

An experiment was done to determine if a gene for photoperiod insensitivity occurs on the S14 translocated segment as was suggested by Marais *et al*. (2005). The experiment was done in a controlled environment chamber (18-23°C) by exposing CS, CS-S, W84-17 as well as CS-S14 (+) and CS-S14 (-) plants to a short day and a long day treatment in order to compare the differential light response of these genotypes. Genotypes CS-S14 (+) and CS-S14 (-) are, respectively, leaf rust resistant and leaf rust susceptible segregates from a CS near isogenic line segregating for the S14 translocation (Table 2.8).

Table 2.8 Genotypes used in an experiment to compare their response to two light regimes.

| No. | Genotype | Description |
|---|---|---|
| 1 | CS | Common wheat (tall) |
| 2 | CS-S | Semi-dwarf near isogenic line of CS that has *Rht-B1b* (from Inia 66) on chromosome 4BS. Pedigree = Inia 66/7*CS |
| 3 | W84-17 | Semi-dwarf (*Rht-B1b* – chromosome 4BS) common wheat |
| 4 | CS-S14 | Common wheat line segregating for a 2DL translocation from *Ae. kotschyi*. Pedigree = CSM2D// *Ae.kotschyi*-617/ 4*CS/3/CS-S |

In the long day treatment the plants received 14 hours of high intensity illumination (approx. 260 µmol $m^{-2}s^{-1}$) followed by 10 hours of darkness while the short day treatment consisted of 8 hours of high intensity illumination (approx. 260 µmol $m^{-2}s^{-1}$) followed by 16 hours of darkness. Each treatment consisted of eight plants for each genotype except for CS-S14 (+) (seven plants) and CS-S14 (-) (four plants). Seeds of each genotype were germinated and the seedlings inoculated with leaf rust pathotype UVPrt8. This was done to identify CS-S14 plants with (+) and without (-) the translocation. The seedlings were then transferred to pots (14 cm in diameter) containing a 50/50 mixture of sand and peat. The pots were placed randomly under the lights and re-randomized on a regular basis. The days from the start of the experiment until flowering were calculated for each plant.

### 2.6 Leaf rust seedling resistance screening

#### 2.6.1 Inoculation

Seedlings were inoculated at the two leaf stage (approx. 9-12 days old) using leaf rust pathotype UVPrt8. Fresh spores were suspended in distilled water containing a small amount of wetting agent (Tween 20) and sprayed onto the seedlings using a fine nozzle trigger sprayer. High humidity is needed for spore germination and infection and seedlings were therefore covered with a moistened clear plastic bag and incubated in the dark at 18-23°C for 24 hours. Following incubation, the plastic bags were removed and the plants were placed in a growth chamber with a temperature of 18-23°C and a day/night cycle of 14/10 hours.

#### 2.6.2 Resistance scoring

Seedlings were scored 10-15 days after inoculation using a 0-4 scale described by Roelfs *et al.* (1992). The infection types were rated as follows: 0 = Immune with no visible uredia, ; = very resistant with the appearance of hypersensitive necrotic areas, 1 = resistant where small uredia were associated with necrosis, 2 = moderately resistant where chlorosis or necrosis surrounded small/medium sized uredia, 3 = moderately susceptible where medium sized uredia, which may be associated with necrosis were visible, 4 = susceptible where large uredia with no chlorosis were observed. The symbols + (uredinia larger than average) and – (uredinia smaller than average) were used to describe variation within each infection type with ++ and = indicating the upper and lower levels, respectively.

### 2.6.3 Spore collection, maintenance and storage

A susceptible triticale breeding line, 'KCK', was used to maintain and increase leaf rust pathotype, UVPrt8, obtained from the Department of Plant Pathology, UFS. Infections were done employing fresh spores collected off diseased plants. For long term storage, spores were collected on a clean piece of paper by shaking them from infected leaves. These spores were dried on silica gel, placed in eppendorf tubes and stored at -80°C. Prior to use, the stored spores were subjected to a heat shock treatment (37°C, 10 min).

## 2.7 Root tip chromosome counts

Ten to 15 kernels were evenly spread on moistened Whatman filter paper in a Petri dish and covered with cling wrap to prevent the loss of moisture. Petri dishes were incubated at room temperature (21°C) for 2-3 days after which the seminal roots were cut. One or two seminal roots were cut from each kernel when 1-2 cm in length and placed in numbered glass vials (18mm x 50mm) with cold $dH_2O$. The seedlings were placed on moistened filter paper in identically numbered Sterilin dish compartments and stored at 4°C for up to two weeks. The vials containing the root tips were placed on ice and incubated at 4°C for 29 hours after which the roots were fixed with freshly made fixative (3 parts methanol : 1 part propionic acid) for 1-2 weeks (minimum of two days) at room temperature before staining. Following fixation the fixative was replaced with $dH_2O$ and incubated at room temperature for 30 min after which the root tips were carried over to vials containing pre-heated 1N HCL in a heating block at 60°C for 7½ min. Following DNA hydrolysis the root tips were rinsed with $dH_2O$ for 1-2 minutes where after the $dH_2O$ was replaced with leuco-basic fuchsin, prepared as described by Darlington and La Cour (1960), and incubated at 4°C for two hours or more. Following incubation the leuco-basic fuchsin was discarded and the root tips washed twice with $dH_2O$. The root tips were rinsed with a 7.5 mM sodium acetate buffer solution (pH 4.5) which was replaced with a 2½% (w/v) pectinase solution (0.5 g of pectinase from Serevac dissolved in 20 ml of the sodium acetate solution described above) and incubated at 37°C for 20 min. The pectinase solution was replaced with $dH_2O$ after 20 min and the root tips stored at 4°C until mounting.

A root tip was placed on a clean slide and the stained tip cut off in a drop of Rosner 1% (w/v) aceto-carmine which contained an equal volume of propionic acid for improved

chromosome spread. The cells were released from the root tip when gently tapped with the blunt end of a wood peg. The cells were covered with a cover glass and the slide placed inside a piece of folded blotting paper. Firm pressure was applied on top of the slide to remove redundant aceto-carmine and to ensure the sufficient spread of chromosomes before they were studied under the 100X oil-immersion lens of a microscope.

**2.8 Genomic DNA extractions and quantification**

Extractions were performed using the protocol of Doyle and Doyle (1990) with slight modifications. Approximately 0.1 g of young leaf tissue was cut into small pieces in a 2.2 ml microfuge tube. Three ball bearings (1.0 mm) and 800 µl of pre-heated 2% (w/v) CTAB extraction buffer (1.4 M NaCl, 20 mM $Na_2EDTA$ (pH 8.0), 100 mM Tris-Cl (pH 8.0) and 0.2% (v/v) β-mercaptoethanol) were added to each sample which was ground for 3 min at 30 Hz using a tissuelyser (Qiagen). The samples were incubated in a waterbath at 60°C for 60 min after which 800 µl chloroform-isoamyl alcohol (24:1) was added to the tube, mixed gently and centrifuged at 12,000 rpm for 8 min. The aqueous upper phase was transferred to a clean 2.2 ml microfuge tube and one volume of phenol chloroform-isoamyl alcohol (25:24:1) was added, inverted and centrifuged at 12,000 rpm for 3 min at room temperature. The supernatant was transferred to a new 2.2 ml microfuge tube, extracted with one volume of chloroform-isoamyl alcohol (24:1) and centrifuged at 12,000 rpm for 5 min at room temperature. The aqueous phase was carried over to a new 2.2 ml microfuge tube and the DNA precipitated using one volume of cold isopropanol. Samples were incubated at -20°C for at least 60 min or overnight if possible. Following incubation samples were centrifuged at 12,000 rpm for 10 min at 4°C and the pellet washed with 500 µl of cold 70% ethanol at 12,000 rpm for 5 min at 4°C. The 70% ethanol was discarded and the pellet allowed to air dry after which it was dissolved in 50 µl of TE (10 mM Tris-Cl (pH 8.0) and 1 mM $Na_2EDTA$ (pH 8.0)) containing 40 µg/ml Rnase A and incubated for 30 min at 37°C. The samples were allowed to cool down and DNA was precipitated with 1/10 volume of 3 M sodium acetate (pH 5.0) and 2 volumes of cold ethanol and centrifuged at 12000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet washed twice with 500 µl of cold 70% ethanol at 12,000 rpm for 5 min at 4°C. The 70% ethanol was discarded and the pellet allowed to air dry after which it was dissolved in 20-50 µl of $dH_2O$.

A Nanodrop® ND-1000 spectrophotometer was used to determine the DNA concentration of the samples. The Nanodrop® was calibrated with $dH_2O$ after which two µl of the DNA sample was loaded and the concentration determined.

**2.9 Making of crosses**

Florets were emasculated as soon as the spikes emerged and covered with a glycine bag to avoid cross pollination. Florets were cut open after 2-6 days (depending on the weather) and pollen, collected from mature anthers of the male parent, was dusted onto the florets using tweezers. Each pollinated spike was then tagged with a tag containing information on the parents.

# Chapter 3

# RESULTS AND DISCUSSION

## 3.1 The identification of translocation recombinants

In order to identify plants containing the *Lr54* translocation, a total of 468 $TF_1$ (04M144) seeds were germinated and 419 seedlings were eventually screened for leaf rust resistance using *P. triticina* pathotype UVPrt8. Two hundred and seventy six (58.97%) seedlings were resistant. Nine RFLP markers, that map to the extremities of chromosome arm 2DL, were evaluated in a first attempt to identify translocation recombinants. It was anticipated that RFLPs associated specifically with the translocation would be detected and that these could be used as dominant markers to select for recombinants. The nine RFLP probes were therefore hybridized to restriction digests of *Ae. kotschyi*, CS-S14 plants heterozygous for the translocation, W84-17 and CS lines that were nullisomic for chromosomes 2A (tetrasomic 2B), 2B (tetrasomic 2A) and 2D (tetrasomic 2A), respectively, to determine if they can detect *Ae. kotschyi* polymorphisms and to confirm their chromosome location.

When the nine RFLP probes were tested on the plant panel the data obtained with the CS nulli-tetrasomics confirmed that six probes hybridized to restriction fragments from the homoeologous group 2 loci whereas two probes (PSR151, KSU H16) could not be scored as a result of weak hybridization signals (Fig. 3.1). Probe PSR571 hybridized to similar sized fragments in all the wheat genotypes, suggesting that either it could not differentiate among group 2 homoeo-alleles or it detected a locus that was not associated with the group 2 chromosomes. None of the probes detected a fragment that was uniquely associated with *Ae. kotschyi* and the translocation derived from it. Thus, none of the nine RFLP markers were suited for the detection of translocation recombinants. This disappointing result as well as a paucity of further RFLP markers that map to the 2DL proximal and distal regions necessitated rethinking of the screening strategy.

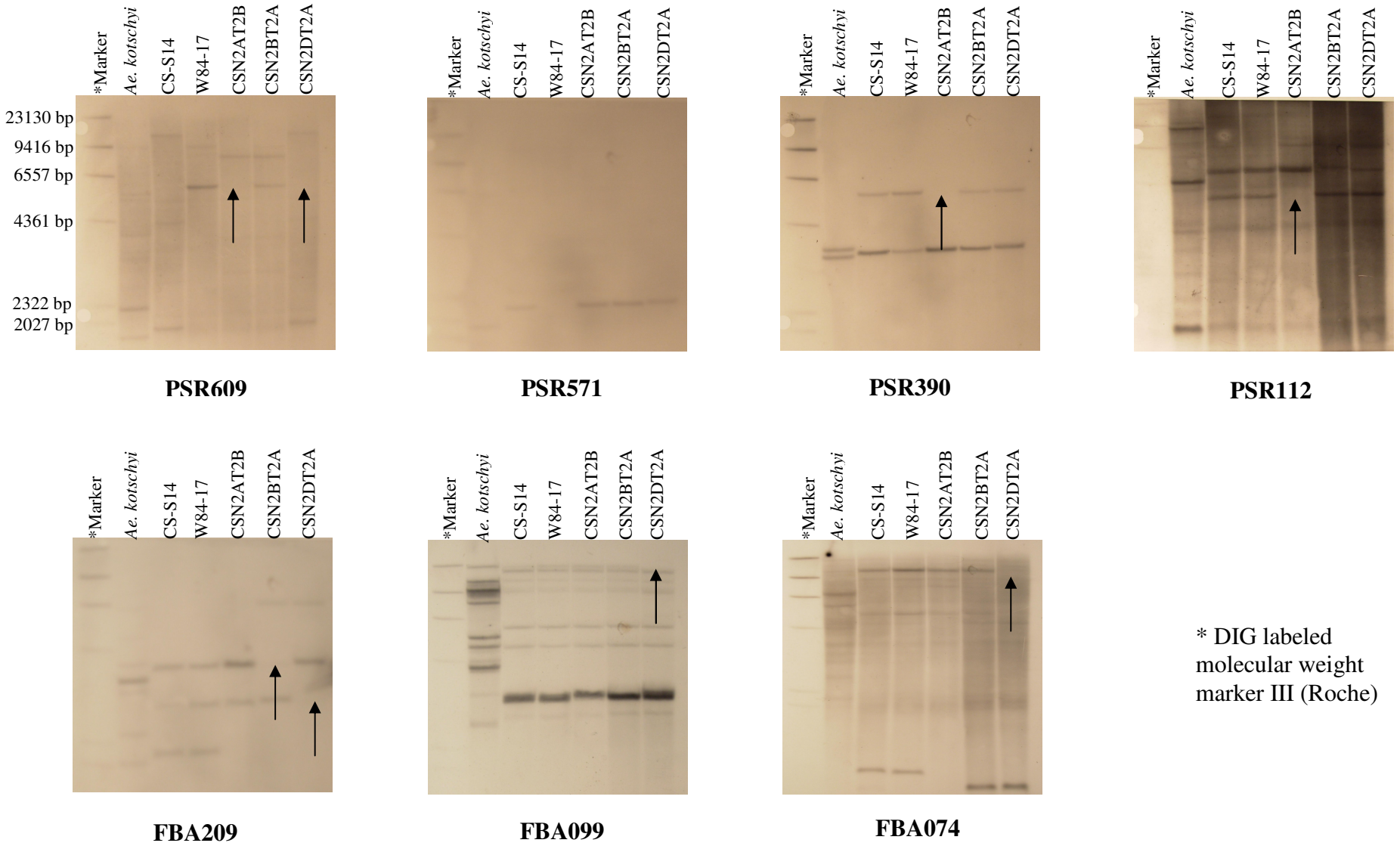



Figure 3.1 Autoradiographs obtained following RFLP analysis of a test panel of genotypes making use of nine 2DL-specific probes. This was done in an attempt to identify alleles uniquely associated with *Ae. kotschyi* and the S14 translocation that could be used for the identification of translocation recombinants. The arrows indicate loci that occur on homoeologous group 2 chromosomes of wheat and serve to confirm the chromosome location of the marker loci.

Since well developed microsatellite maps were becoming available at that stage of the study, accompanied with ready access to primer information and amplification conditions, it was decided to switch to the use of microsatellite markers. Microsatellites are mostly chromosome specific and the primers often do not amplify an *Ae. kotschyi* derived fragment. Thus, wheat microsatellite primers generally produce an amplified fragment(s) that serves as a dominant marker for the presence of the wheat locus, whereas the corresponding homoeologous region on the S14 translocation will not amplify, thus resulting in a null allele. The null allele can therefore be used as a indicator of the presence of alien chromatin, yet in order to analyze the recombinants in this manner it was necessary to either develop translocation homozygotes or hemizygotes. The simplest solution appeared to be to cross each of the 276 resistant testcross progenies with the CS nullisomic 2D tetrasomic 2A/2D stocks and to select resistant hemizygotes among the $F_1$. Each resistant $TF_1$ (04M144) plant was therefore crossed as the male parent with CS nullisomic 2D tetrasomic 2A/2B. A high level of sterility among the resistant $TF_1$ plants made crossing difficult and a total of 193 $F_1$ hybrids were produced and were tested for seedling leaf rust resistance using *P. triticina* pathotype UVPrt8. The number of seeds obtained for each cross was extremely low, in some instances only a single seed was produced, and 159 resistant $F_1$ hybrids were recovered following leaf rust screening. DNA was extracted from these plants.

Seven microsatellite markers, specific for chromosome arm 2DL were chosen from existing molecular and physical maps (Fig. 2.2). These markers were first tested on a genotype panel (Table 2.1) to confirm their chromosome arm location and to study the polymorphisms produced among *Ae. kotschyi*, W84-17 and CS. Two markers, Wmc144 and Wmc18, were found to detect loci on chromosome arm 2DS rather than on 2DL. One marker, Cfd239, could not adequately distinguish the parental genotypes as it amplified a locus in CS but detected null alleles in *Ae. kotschyi*, CS-S14 (hemi) and W84-17. Another marker, Gwm301, was not used due to the complexity of the profiles that amplified in *Ae. kotschyi*, CS-S14 (hemi), CS and W84-17. Each of the three remaining markers mapped to chromosome arm 2DL and produced amplification products in both CS and W84-17 but null-products in *Ae. kotschyi*. These three marker loci, *Xbarc228* (proximal), *Xwmc167* and *Xgdm6* (distal) were used to screen the $F_1$ hybrids. The polymorphisms produced by the three markers on the genotype panel of Table 2.1 are summarized in Fig. 3.2.

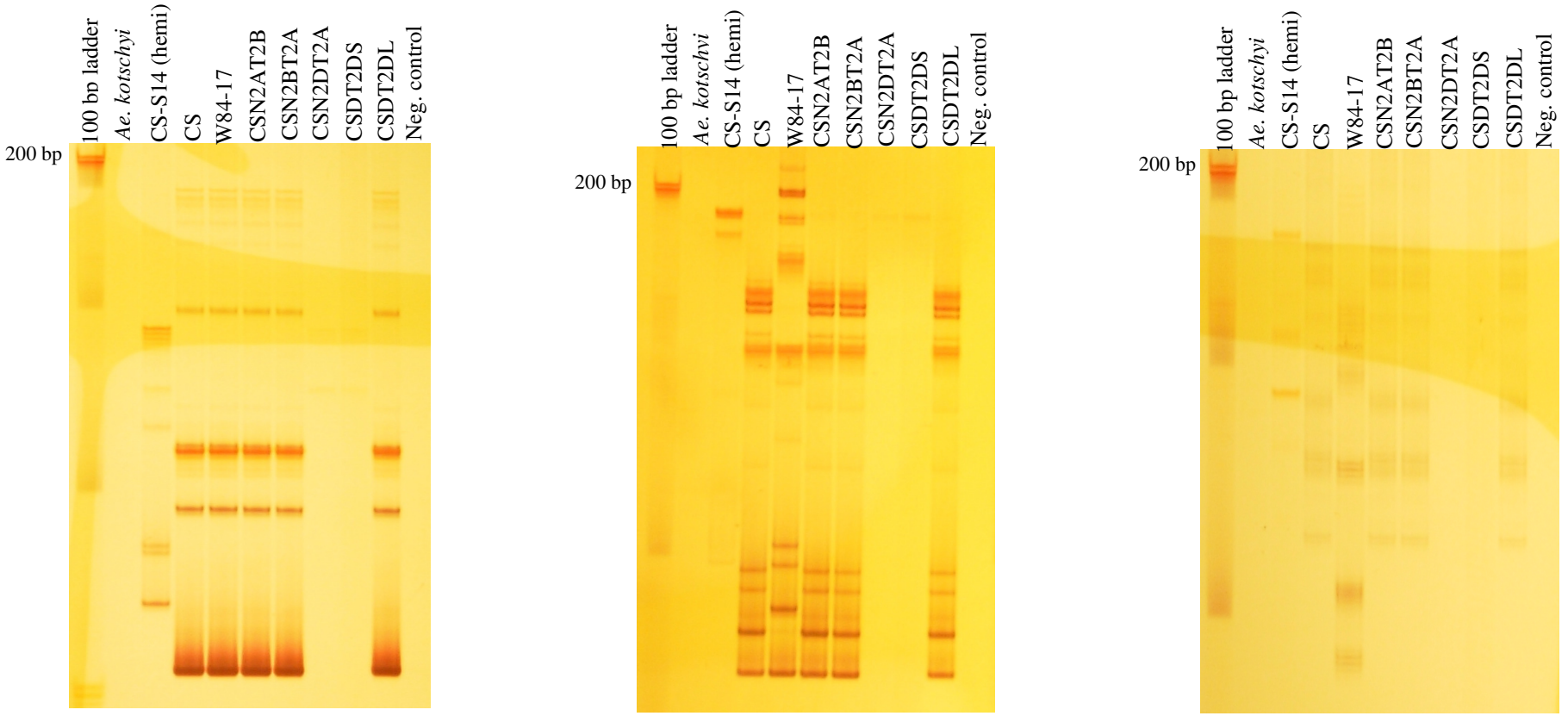



Figure 3.2 Microsatellite amplification products produced with the test panel of genotypes. PCR products were separated on a 6% poly-acrylamide denaturing gel and visualized by silver staining. The microsatellite markers shown were used to screen resistant $F_1$ hybrids in an attempt to identify translocation recombinants.

The three selected microsatellite markers were specific for chromosome arm 2DL since prominent bands were amplified in CS, W84-17 and CS ditelosomic 2DL plants but not in CS ditelosomic 2DS or CS nullisomic 2D tetrasomic 2A plants. Markers Barc228 and Gdm6 amplified different alleles in CS and W84-17, whereas Wmc167 amplified the same allele in CS and W84-17. *Aegilops kotschyi* did not produce any PCR product from the three primer sets, whereas some amplification was seen in CS-S14 (hemi). The latter bands were absent in wheats lacking 2DL. The same bands were subsequently (while screening the testcross progeny) seen in plants hemizygous for the translocation. While their origin is not clear the bands were distinctly different from the diagnostic bands and did not affect characterization.

Following the screening of 159 resistant $F_1$ hybrids with the three microsatellite markers (Addendum A), 10 lines appeared to be recombinants as they showed absence of certain microsatellite loci but presence of others, whereas 99 lines had the *Ae. kotschyi* parental genotype and thus were not recombinants. However, 50 lines expressed the wheat genotype at all three marker loci as though they were recombinants. It was extremely unlikely that all of these plants could be recombinants and that each retained the resistance genes on a relatively small segment of *Ae. kotschyi* chromatin. It was therefore necessary to either verify the data or to find an alternative explanation for the origin of the 50 apparently recombined plants. Three possible scenarios that could explain the latter results seemed evident and were therefore investigated:

**(A)** The data could be correct. This would mean that homoeologous pairing did in fact occur between the translocation on chromosome arm 2DL and its wheat homoeologue, thus resulting in testcross $F_1$ plants containing the recombined translocation (Fig. 3.3).

**(B)** A second translocation to another unknown chromosome may have occurred in one of the 03M130 plants (CS-S14/2*CS *ph1b* mutant) that was produced by Marais *et al*. (2005). This may have resulted in a large number of plants hemizygous for chromosome $2D^{W84\text{-}17/CS}$ (Fig. 3.4).

**(C)** The unexpected presence of a chromosome 2D in the CS nullisomic 2D line used as male parent or accidental crossing with a line containing the normal chromosome 2D may have given rise to plants heterozygous for the translocation (Fig. 3.5).

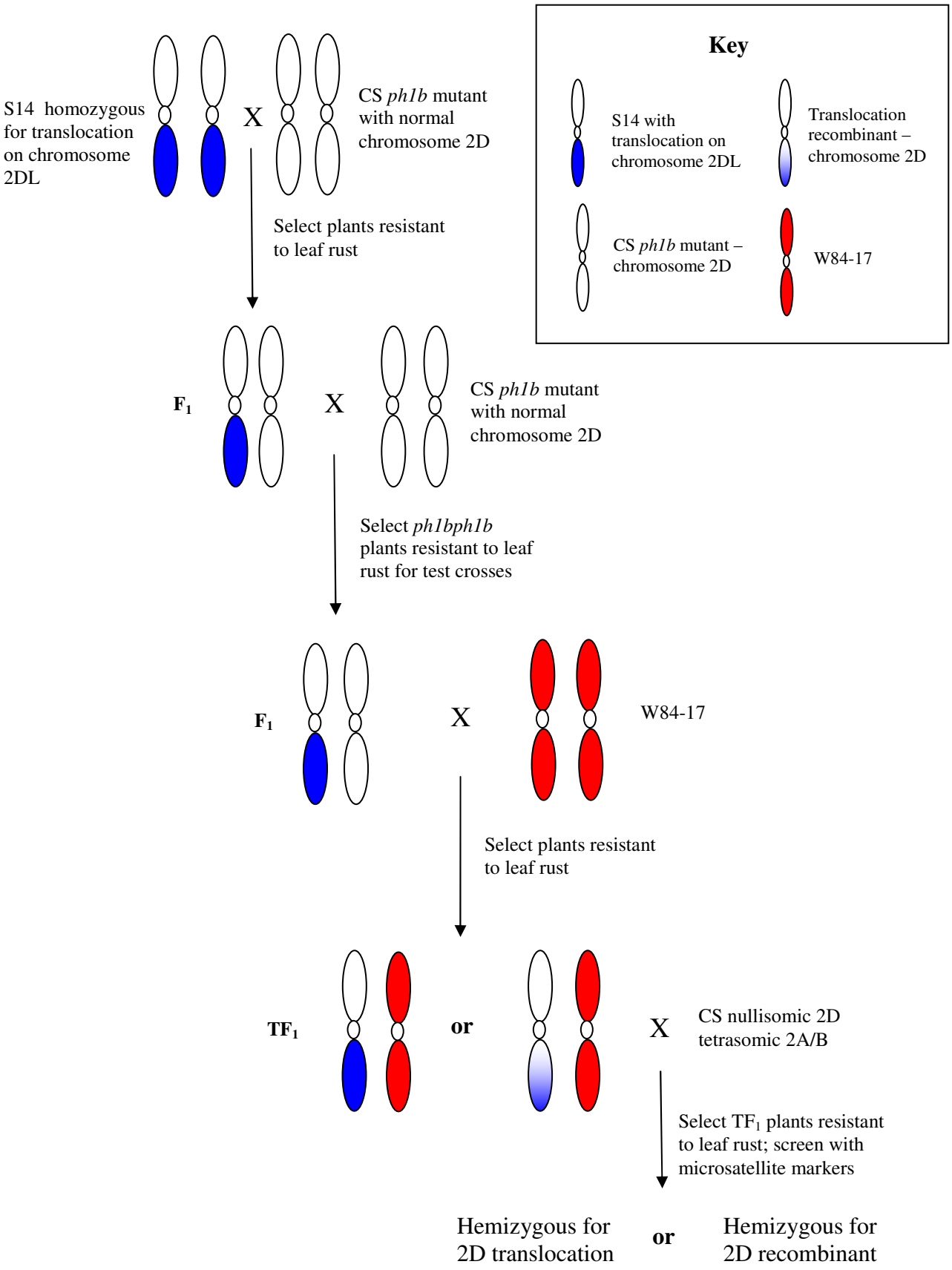
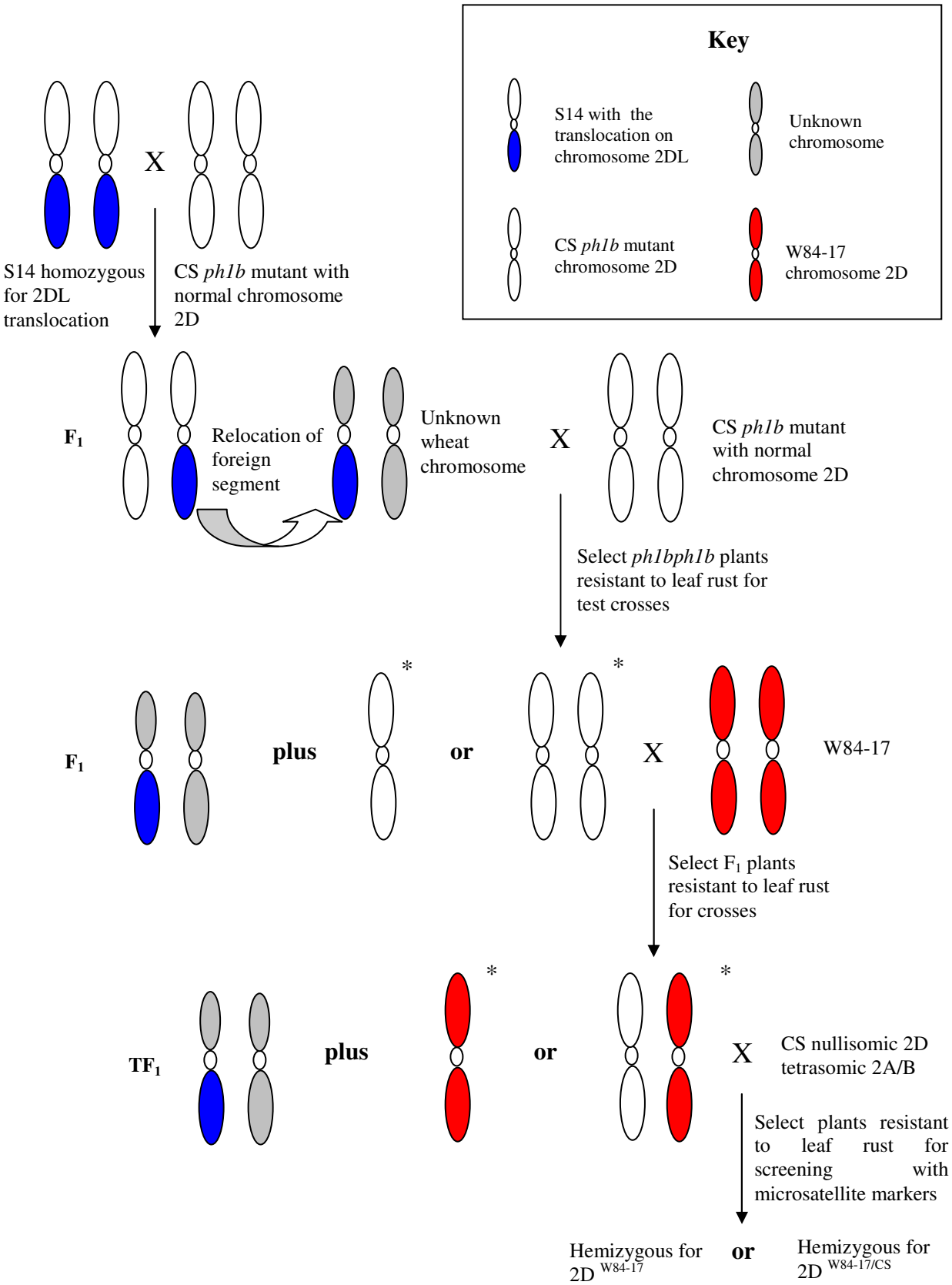



Figure 3.3 The chromosome 2D products that would have resulted had normal homoeologous recombination occurred between the S14 translocation on chromosome 2DL and its wheat homoeologue. The diagram outlines all the events and genotypes involved from the beginning of the homoeologous pairing induction experiment until the production of the hemizygous testcross progeny.




* The type of translocation that could have taken place during meiosis is unknown. If it was a Robertsonian translocation then the $F_1$ could have been mono- or disomic for chromosome 2D.

Figure 3.4 The chromosome 2D products that would have resulted if the S14 translocation was relocated (in one of the plants) to another (unknown) chromosome prior to the onset of the homoeologous pairing induction experiment. The diagram outlines all the events and genotypes involved from the beginning of the experiment.

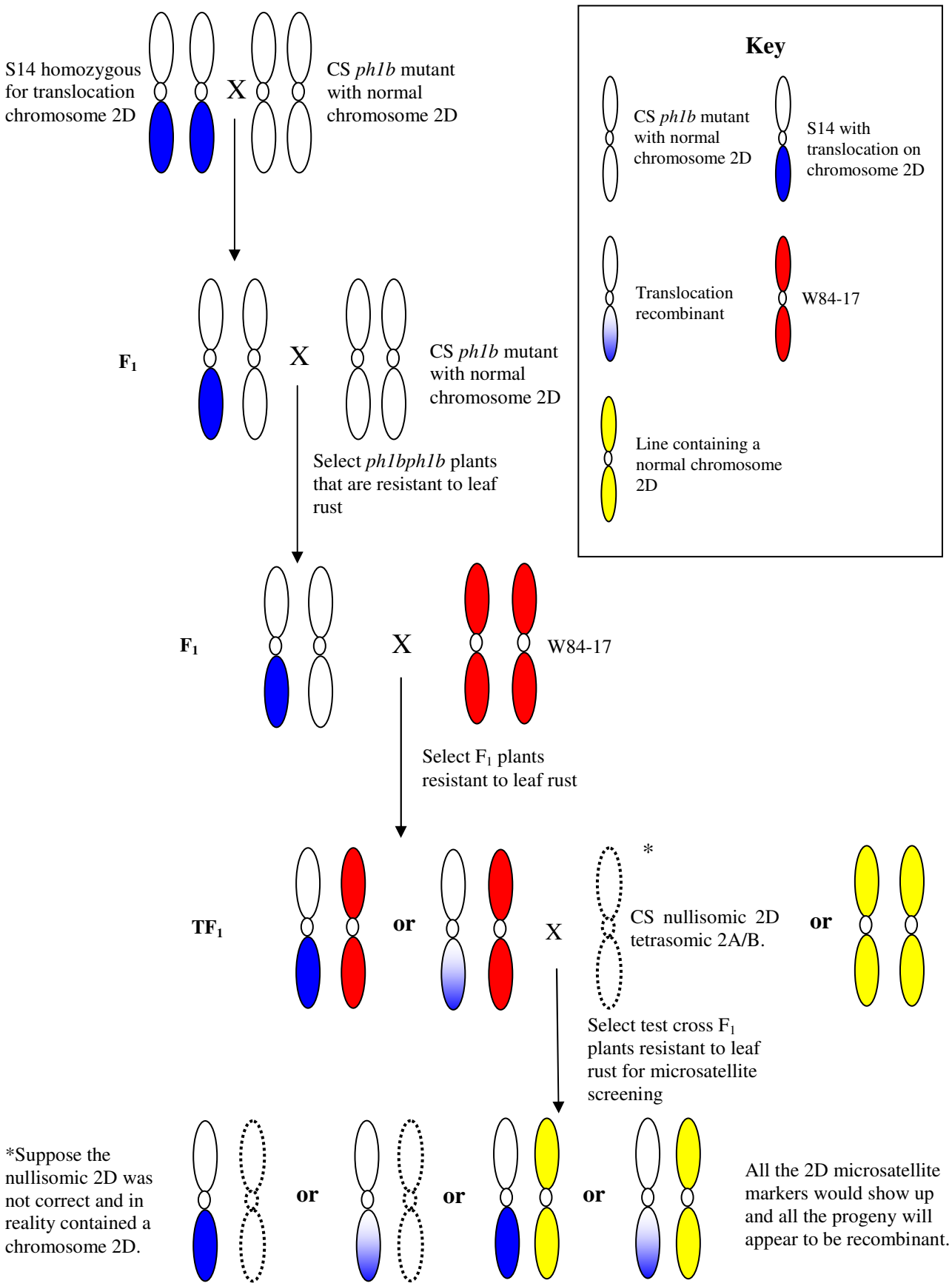



Figure 3.5 The chromosome 2D products that would have resulted had the CS nullisomic 2D tetrasomic 2A/2B line contained an unexpected chromosome 2D or when crosses were conducted with a line having a normal chromosome 2D. The diagram outlines all the events and genotypes from the beginning of the homoeologous pairing induction experiment until the production of the heterozygous testcross progeny.

In **scenario A** the events of normal recombination between the translocation and its wheat homoeologue in the pairing induction experiment is reviewed (Fig. 3.3). Recombination between the translocation and the wheat homoeologue would have resulted in testcross $F_1$ plants heterozygous for the recombined translocation or heterozygous for the non-recombined translocation. If scenario A proved to be correct then the resistance locus in the 50 resistant plants that expressed the dominant wheat microsatellite alleles at all three loci must still map to chromosome 2D. Also, the recombinants could have expressed either the CS or W84-17 alleles of *Xbarc228* and *Xgdm6*, but mostly the CS alleles. Screening of the resistant $F_1$ hybrids showed that both the CS and W84-17 alleles of *Xbarc288* and *Xgdm6* were expressed in the 50 'recombinant' lines. The W84-17 alleles of *Xbarc228* and *Xgdm6* occurred in 39 of the 50 'recombinants'. While it was highly unlikely that so many recombinants could have been obtained, it is not totally impossible in view of the segregation distortion associated with the transmission of the S14 translocation in certain genetic backgrounds (Marais *et al*., 2005). Marais (1990) and Prins and Marais (1999) showed that interaction of multiple genetic factors present in the wheat genome and on the *Lr19* translocation determined both the severity and direction of segregation distortion. Thus, in the present material genetic interactions may similarly have been responsible for the preferential transmission of specific gametic genotypes. However, while segregation distortion was evident in the present material these effects appeared to be mild and seemed to be an unlikely cause of the high number of 'recombinants' that were encountered. To determine whether the plants were true recombinants it would have been necessary to first of all confirm that the translocation was still associated with 2DL.

In **scenario B** the effect that a second translocation to an unknown chromosome may have had on the outcome of the pairing induction experiment is reviewed (Fig. 3.4). The translocation may have occurred in one of the plants following the crosses of CS-S14 with the CS *ph1b* mutant. However, the type of translocation would be unknown. If it were a Robertsonian translocation, the $F_1$ could have been monosomic or disomic for chromosome 2D and testcrosses with W84-17 would have produced plants monosomic for chromosome 2D having the W84-17 genotype or plants disomic for chromosome 2D having the CS and W84-17 genotypes. If scenario (B) is true then the resistance would also no longer map to 2D

but most likely (although not necessarily) to one of the group 2 homoeologues. The 50 plants that expressed the wheat genotype at all three marker loci would have had either the CS or W84-17 alleles of *Xgdm6* and *Xbarc228,* as was in fact the case.

In **scenario C** the effect of a chromosome 2D in CS nullisomic 2D or a plant containing a normal chromosome 2D might have on the outcome of the pairing induction experiment is outlined (Fig. 3.5). If normal recombination occurred between the translocation and its wheat homoeologue plants heterozygous for the recombined translocation and plants heterozygous for the non-recombined translocation would have been produced. The crossing of these lines with CS nullisomic 2D that in reality contained a chromosome 2D or a line containing a normal chromosome 2D would have produced plants disomic for chromosome 2D. If scenario (C) was true then the 50 resistant plants would have expressed the CS alleles of *Xgdm6* and *Xbarc228* or the genotype of the plant that contributed the extra 2D chromosome. In this case the resistance will obviously still map on chromosome 2D.

Monosomic analysis was therefore conducted to determine the chromosome location of the translocation in the 50 unconfirmed 'recombinants'. CS nullisomics for chromosomes 3A, 7A, 7B and 7D as well as monosomics for the remaining 17 chromosomes were pollinated with a representative, unconfirmed 'recombinant' (line 04M144-16). Monosomic $F_1$ hybrid plants were selected using root tip chromosome counts and were grown in a greenhouse. Twenty five $F_2$ seeds from each monosomic plant were screened for resistance to *P. triticina* pathotype (UVPrt8) and the segregation ratio of the resistance was determined (Table 3.1).

While the expected $F_2$ segregation ratio of a single dominant resistance gene in a disomic plant is 3:1, an $F_2$ segregation ratio of approximately 97 resistant: 3 susceptible plants can be expected when the resistance gene occurs on a monosomic chromosome (Law *et al*., 1987). The probability that the $F_2$ data fit a 3:1 Mendelian segregation ratio was calculated for each chromosome ($\alpha = 0.05$) and only the $F_2$ derived from the chromosome 7B monosomics did not conform to a 3:1 ratio.

Table 3.1 Results of a monosomic analysis of the leaf rust resistant line, 04M144-16. The $\chi^2$ value and the probability (P) that the observed segregation ratio conformed to 3:1 was also calculated for each chromosome.

| Chromosome | Resistant | Susceptible | $\chi^2$ value (3:1) | P-value |
|---|---|---|---|---|
| **1A** | 18 | 5 | 0.130 | 0.718 |
| **1B** | 19 | 5 | 0.222 | 0.637 |
| **1D** | 15 | 5 | 0 | 1 |
| **2A** | 18 | 6 | 0 | 1 |
| **2B** | 16 | 7 | 0.362 | 0.547 |
| **2D** | 17 | 7 | 0.222 | 0.637 |
| **3A** | 15 | 9 | 2 | 0.157 |
| **3B** | 13 | 9 | 2.970 | 0.085 |
| **3D** | 18 | 6 | 0 | 1 |
| **4A** | 16 | 7 | 0.362 | 0.547 |
| **4B** | 11 | 4 | 0.022 | 0.881 |
| **4D** | 18 | 6 | 0 | 1 |
| **5A** | 19 | 5 | 0.222 | 0.637 |
| **5B** | 20 | 5 | 0.333 | 0.564 |
| **5D** | 17 | 8 | 0.653 | 0.419 |
| **6A** | 15 | 10 | 3 | 0.083 |
| **6B** | 18 | 7 | 0.120 | 0.729 |
| **6D** | 19 | 6 | 0.013 | 0.908 |
| **7A** | 17 | 7 | 0.222 | 0.637 |
| **7B** | 25 | 0 | 8.333 | 0.004 |
| **7D** | 16 | 8 | 0.889 | 0.346 |

Since it seemed unlikely that *Lr54* would have been translocated to a chromosome that does not belong to homoeologous group 2, the possibility that the gene in 04M144-16 is not *Lr54* was investigated. The *Lr19* gene produces a similarly strong infection type and a recombinant form of *Lr19*, referred to as *Lr19*-149, was developed by Marais (1992) and mapped to chromosome arm 7BL (Prins *et al*. 1997). This line was grown in the green house while the allosyndetic pairing experiment was being conducted and could have been the source of the aberrant results. Prins *et al*. (2001) developed a highly specific STS marker, $STSLr19_{130}$, that amplifies a 130 bp fragment in lines carrying *Lr19* (Fig. 3.6). This marker was used to screen the 50 deviant lines. The results obtained with 10 of the 50 lines are shown in Fig. 3.6 whereas the data of the remaining 40 lines are given in Addendum B.

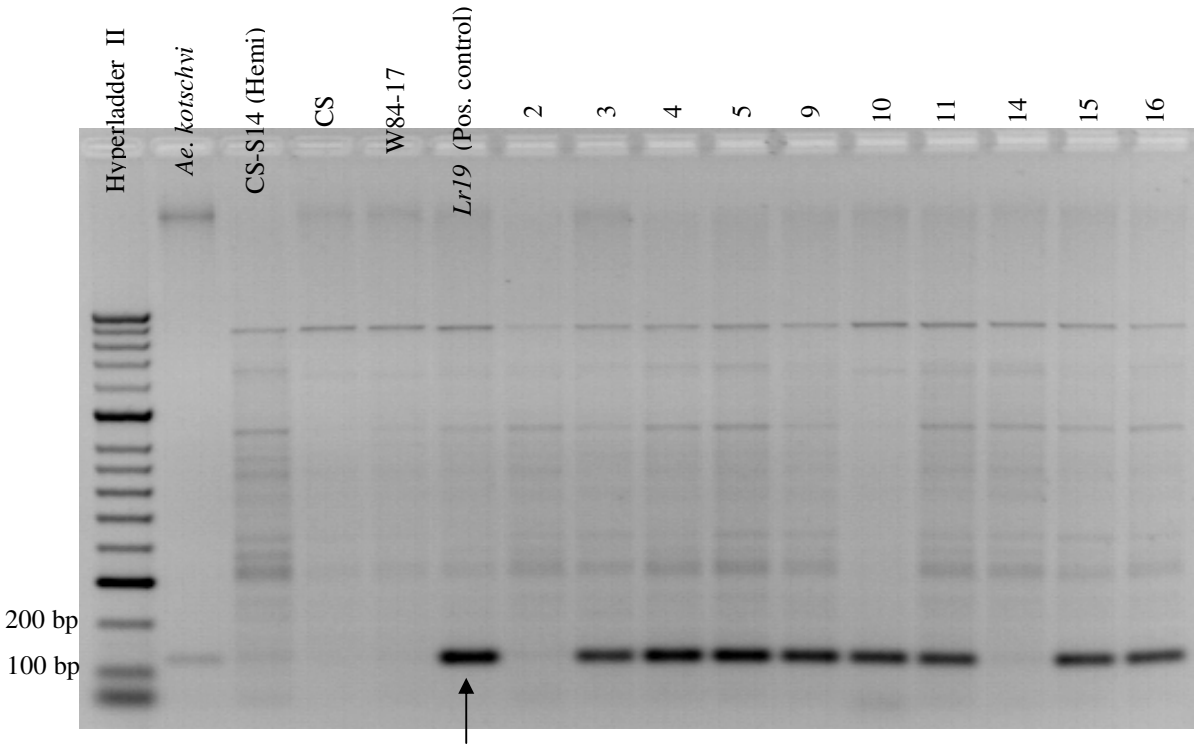



Figure 3.6 Marker $STSLr19_{130}$ was used to screen for the presence of *Lr19* in 50 lines that initially appeared to be *Lr54* recombinants. This gel photo shows the control panel and 10 of the 50 lines that were screened. The arrow indicates the 130 bp band amplified in lines containing *Lr19*.

Marker $STSLr19_{130}$ amplified a prominent band (130 bp) in lines carrying *Lr19* whereas a less intense band was associated with lines that does not have *Lr19*. Fifteen of the 50 lines had *Lr19* whereas the remaining lines lacked *Lr19*. The presence of *Lr19* among the recombinants suggested that instead of having pollinated CS nulli 2D

tetra 2A/B with the $F_1$: 04M144 plants, plants with the *Lr19* translocation were mistakenly used as female parents to produce the 50 families in question. This also means that the testcross $F_1$ thus produced were disomic rather than hemizygous 2D. Resistant testcross $F_1$ were selected and used to provide DNA for the *Lr19* tests and both plants with *Lr19* (with or without *Lr54*) and without *Lr19* (but having *Lr54*) were retained (Fig. 3.6) which suggested that a line heterozygous for the *Lr19* translocation was used in the testcross. Thus, as the data of the 50 deviant lines were confounded by the presence of *Lr19* as well as a normal 2D chromosome, these were not analyzed further.

In total, 109 testcross $F_1$ plants remained. Each of the plants were confirmed with the microsatellite genotyping (three microsatellite loci) as being hemizygous for chromosome 2D and having either the complete or recombined S14 translocation. Ten of the plants proved to have a recombined translocation. Linkage analysis was conducted on data from 109 plants (Table 3.2) to determine the order of three microsatellite markers (Fig. 3.7).

Table 3.2 A summary of the microsatellite data obtained for 109 $TF_1$ plants that had either the complete or a recombined translocation.

| **Line** | ***Xbarc228*** | ***Xgdm6*** | ***Xwmc167*** |
|---|---|---|---|
| Wheat genotype | + | + | + |
| *Ae. kotschyi* genotype | - | - | - |
| Rec. #25 | - | - | + |
| Rec. #37 | - | + | + |
| Rec. #74 | - | + | + |
| Rec. #119 | - | - | + |
| Rec. #148 | - | - | + |
| Rec. #205 | - | - | + |
| Rec. #247 | - | - | + |
| Rec. #256 | - | + | + |
| Rec. #265 | - | - | + |
| Rec. #273 | - | - | + |
| 99 lines had the *Ae. kotschyi* parental genotype | - | - | - |

The gene order that was derived from the data (*Xbarc228 – Xgdm6 – Xwmc167*) did not correspond with the published map data (Fig. 3.7). The reason for the apparent inconsistency is provided later on in the discussion (section 3.2.1). The calculated distances between loci were 0.03 (*Xbarc228 – Xgdm6*) and 0.06 (*Xgdm6 – Xwmc167*), respectively. However, these are not true map distances but should rather be viewed as allosyndetic map distances. Furthermore, a selected (only resistant plants) data set was used to derive the estimates which imply that they are probably less accurate.

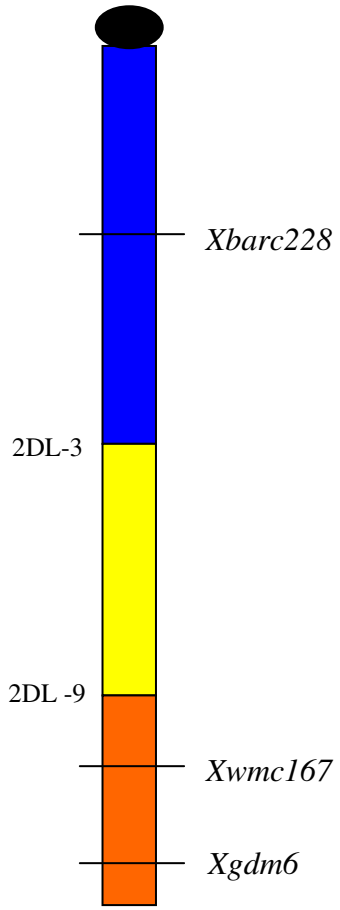



Figure 3.7 A genetic map of chromosome arm 2DL showing the relative locations of three microsatellite loci with respect to the 2DL deletion bins (after Sourdille *et al*., 2004).

The allosyndetic recombination distance between the two distal loci (*Xgdm6* and *Xwmc167*) was roughly double than that between *Xbarc228* and *Xgdm6*. This is in agreement with literature reporting a higher frequency of recombination towards the telomeres of wheat chromosomes (Delaney *et al*., 1995 a, b; Michelson-Young *et al*., 1995; Werner *et al*., 1992). Since only resistant progeny were evaluated, the

frequency of the *Ae. kotschyi* allele among the recombinants at the three loci gave an indication of the relative location of *Lr54*. The frequencies were respectively, *Xbarc228* (100%) - *Xgdm6* (70%) - *Xwmc167* (0%) which would suggest that *Xbarc228* is located closest to *Lr54*. The 10 recombinants were then characterized with further molecular markers in order to determine which had exchanged the largest amount of *Ae. kotschyi* chromatin for wheat chromatin.

## 3.2 Genotyping of 10 recombined translocation chromosomes with the use of further microsatellite markers

A variety of molecular markers (SSR, SCAR, RGA, RAPD and AFLP) were evaluated in an attempt to find markers that map to the chromosome 2DL region where the allosyndetic crossovers occurred. Such markers could be used to: (a) characterize the recombinants and determine the relative amount of foreign DNA associated with each, following which the potentially most useful recombinant could be identified. (b) In addition, the markers were studied in an attempt to find polymorphic loci that occurred on the shortest translocation recombinant and which could eventually be used for marker assisted selection.

### 3.2.1 Use of microsatellite markers to differentiate the 10 recombinants

In addition to the three microsatellite markers that were used to identify translocation recombinants, another seven wheat microsatellite loci, *Xgwm539*, *Xgwm157*, *Xcfd233*, *Xcfd50*, *Xgdm87*, *Xbarc1095* and *Xwmc41* which were reported to occur on 2DL (Röder *et al*., 1998a; 1998b; Pestsova *et al*., 2000; Gupta *et al*., 2002; Guyomarc'h *et al*., 2002; Shi *et al*., 2003; Sourdille *et al*., 2004) were evaluated on the test panel.

Five of the microsatellite markers, Cfd50, Wmc41, Gwm157, Cfd233 and Gwm539 proved to be specific for chromosome arm 2DL since they amplified bands in CS ditelosomic 2DL plants that were absent in CS nullisomic 2D tetrasomic 2A and CS ditelosomic 2DS plants (Fig. 3.8). Markers Gwm539, Cfd233 and Cfd50 amplified different alleles in CS and W84-17, whereas markers Wmc41 and Gwm157 amplified the same allele in CS and W84-17. Markers Gwm539, Gwm157 and

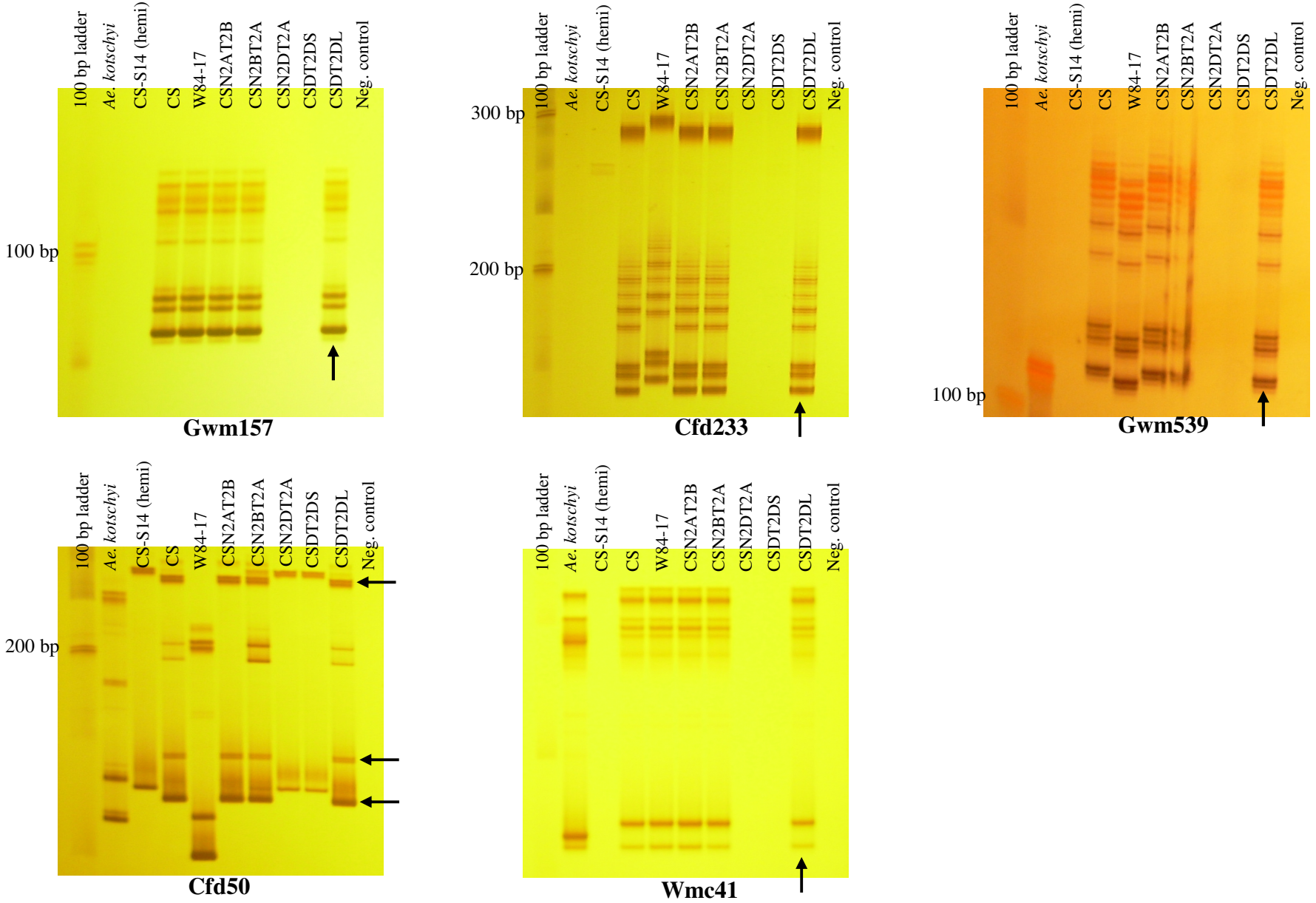



Figure 3.8 Microsatellite bands amplified in the genotypes of the test panel making use of five further primer sets reported to detect SSR loci on 2DL. The 2D polymorphic loci are indicated with arrows.

Wmc41 did not amplify any visible fragments in the S14 hemizygote, whereas Cfd50 amplified two bands in CS-S14 (hemi) that were also visible in the two wheat genotypes that lacked 2DL. Marker Cfd233 also amplified two bands in the S14 hemizygote that were absent in the wheat and *Ae. kotschyi* genotypes. The markers Gwm539, Cfd50 and Wmc41 produced amplification products in *Ae. kotschyi* that were absent in, CS-S14 (hemi) and therefore unrelated to, the translocated segment. The five microsatellite markers were therefore suitable for further characterization of the 10 recombinants, the profiles of which are shown in Addendum C. The results obtained following characterization of the 10 recombinants with microsatellite markers are summarized in Table 3.3.

Table 3.3 A summary of all the chromosome arm 2DL microsatellite data obtained during the identification and characterization of the 10 S14 recombinants.

| Line | *Xbarc 228* | *Xwmc 41* | *Xcfd 233* | *Xgwm 157* | *Xgwm 539* | *Xcfd 50* | *Xwmc 167* | *Xgdm 6* | Crossover positions[1] |
|---|---|---|---|---|---|---|---|---|---|
| *Ae. kotschyi* | - | ● | - | - | - | ● | - | - | |
| CS-S14-hemi | - | - | - | - | - | - | - | - | |
| CS | + | + | + | + | + | + | + | + | |
| W84-17 | +* | + | +* | + | +* | +* | + | +* | |
| Rec. 74 | - | - | - | + | + | +*/+ | + | +* | (iii) |
| Rec. 37 | - | - | - | - | - | +* | + | +* | (i) |
| Rec. 205 | - | - | - | - | - | +* | + | - | (ii) |
| Rec. 256 | - | - | - | - | - | + | + | + | - |
| Rec. 119 | - | - | - | - | - | +* | + | - | (ii) |
| Rec. 148 | - | - | - | - | - | +* | + | - | (ii) |
| Rec. 247 | - | - | - | - | - | +* | + | - | (ii) |
| Rec. 265 | - | - | - | - | - | +*/+ | + | + | (iv) |
| Rec. 273 | - | - | - | - | - | +* | + | - | (ii) |
| Rec. 25 | - | - | - | - | - | +* | + | - | (ii) |

Symbols used: "-" indicates the absence of a wheat allele (= alien chromatin or a deleted region) whereas "+" indicates the presence of a wheat allele; in those instances where CS and W84-17 produced different alleles, "+*" is used to indicate the W84-17 allele; "●" indicates the amplification of fragments in *Ae. kotschyi* that are unrelated to the S14 translocated segment.
[1] Refers to Fig. 3.12 where the positions of possible single crossover events that resulted in the formation of the 10 translocation recombinants are indicated.

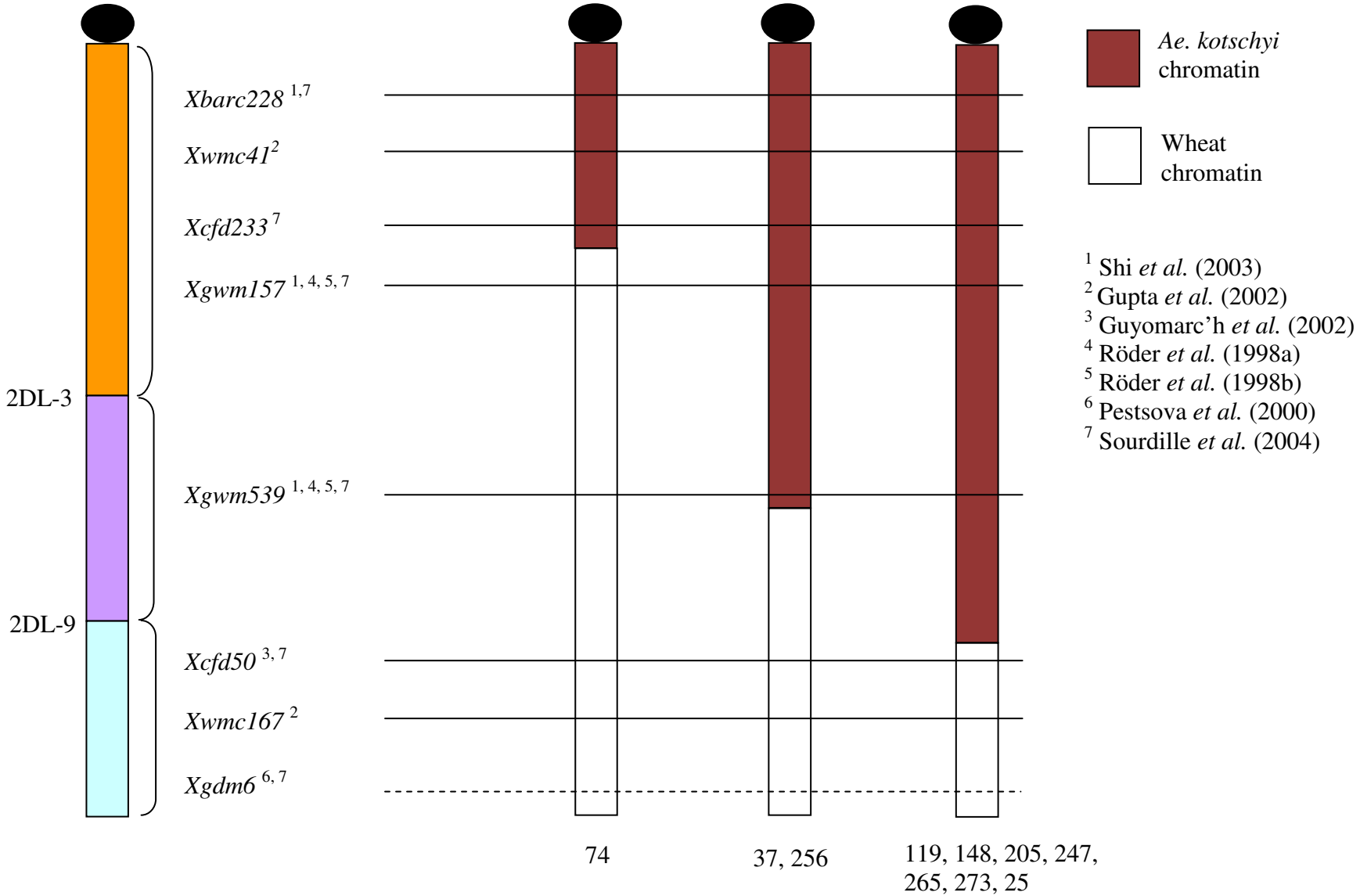



Figure 3.9 Approximate genetic maps (based on published data) of chromosome arm 2DL in 10 *Lr54/Yr37* translocation recombinants. The positions of the microsatellite marker loci employed in this study are shown relative to three deletion bins defined by Sourdille *et al.* (2004). The broken line indicates the location of *Xgdm6* as mapped by Sourdille *et al*. (2004).

The microsatellite marker loci were ordered as suggested in the literature (Fig. 3.9; Table 3.3) and the data could be used to group the recombinants into two size categories. The data obtained with marker locus *Xgdm6* appear to be inconsistent with its mapped location (Sourdille *et al.,* 2004) as six of the recombinants did not produce the wheat allele for this locus. Absence of a wheat allele could either be due to the presence of *Ae. kotschyi* chromatin or deletion of the wheat locus. The presence of *Ae. kotschyi* chromatin would imply that double-crossovers had occurred in six of the ten recombinants which is highly unlikely. Lukaszewski *et al*. (2004) found no multiple exchanges among homoeologous recombinants of chromosomes 2R and 2B. Lukaszewski (1995) studied the distribution of translocation breakpoints following recombination between homoeologous chromosomes of wheat, rye and *Ae. speltoides* and found only one double crossover product among 325 (0.31%) recombined chromosomes. Multiple crossovers also occur at relatively low frequency among homologous chromosomes and may be restricted to the physically longer chromosome arms (Lukaszewski and Curtis, 1993). While the distribution of crossovers between homoeologues is similar to the distribution of crossovers between homologues, the homoeologous exchanges occur at reduced frequency. The data observed for locus *Xgdm6* (Table 3.3) can be more readily explained if its absence in the six recombinants resulted from deletion caused by a chromosome structural difference between the two wheat genotypes CS*ph1b* and W84-17. This will be outlined in detail below.

The data of Table 3.3 show that marker Cfd50 amplified either the CS or W84-17 genotype in eight of the recombinants whereas it amplified both the CS and W84-17 genotype in two recombinants (rec. #74 and rec. #265). Since the recombinants were hemizygous for chromosome 2D (this is confirmed by the fact that the *Ae. kotschyi* genotype occurred at certain marker loci in each recombinant) locus *Xcfd50* must have been duplicated in these two recombinants. The duplication was probably introduced as a result of a chromosome 2DL structural difference between the wheat parents (CS*ph1b* mutant and W84-17). The duplication was confirmed by re-screening the two recombinants with the marker Cfd50 (Fig. 3.10).

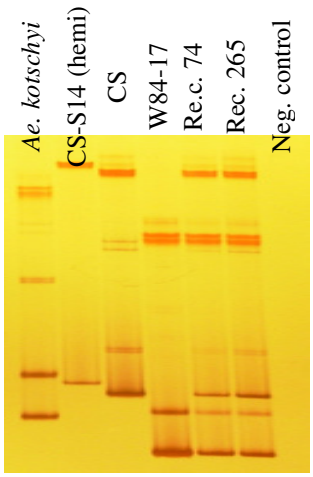



Figure 3.10 Re-screening of recombinants #74 and #265 with microsatellite marker Cfd50.

The microsatellite data in Table 3.3 constitute the outcome of two meiotic events. The first meiotic event involved the chromosome arms of *Ae. kotschyi* and CS whereas the second event involved the chromosome arms of *Ae. kotschyi*/CS and W84-17. The occurrence of CS and W84-17 alleles among the progeny of the second meiotic event, as revealed by markers Cfd50 and Gdm6, showed that the recombinants had recovered normal, functional chromosome 2DL telomeres following single crossovers in the first meiotic event. The meiotic pairing structures that were likely formed during the allosyndetic meiosis are summarized in Fig. 3.11. Probable meiotic pairing structures for the second event between the homoeologous translocation recombinants and the chromosome 2DL arm of W84-17 are summarized in Fig. 3.12. It appears likely that an internal chromosomal translocation of either *Xcfd50* or *Xgdm6* may have resulted in a different locus order in CS*ph1b* and W84-17. During meiosis this difference in locus order would have resulted in the formation of compensation loops in the bivalents formed. Two bivalent configurations were possible and certain crossovers in either of these pairing structures (Fig. 3.12) would have resulted in gametes containing deletions or duplications. The positions where single crossovers could have occurred to produce the recombinants of Table 3.3 are indicated in both Table 3.3 and Fig. 3.12.

### 3.2.2 Potential use of microsatellite loci as recessive molecular markers in marker-aided detection of the shortest recombinant (#74)

The microsatellite loci *Xbarc228*, *Xwmc41* and *Xcfd233* map to the 2DL chromosome region that is occupied by alien chromatin in rec. #74. The alien segment

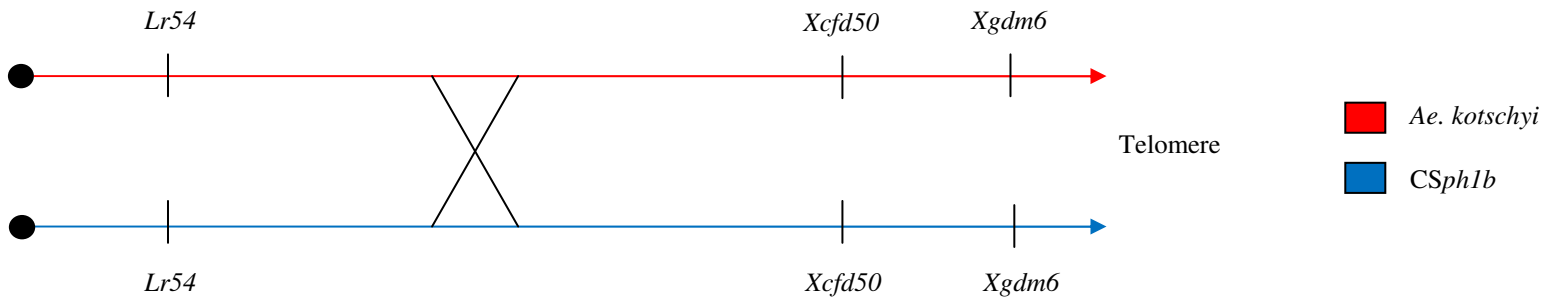



Figure 3.11 First (homoeologous) meiotic recombination event involving chromosome arm 2DL of CS and the translocated *Ae. kotschyi* 2L arm. All the recombinants retained the leaf rust resistance gene (*Lr54*) and recombination between *Lr54* and microsatellite *Xcfd50* locus resulted in 10 recombinants.

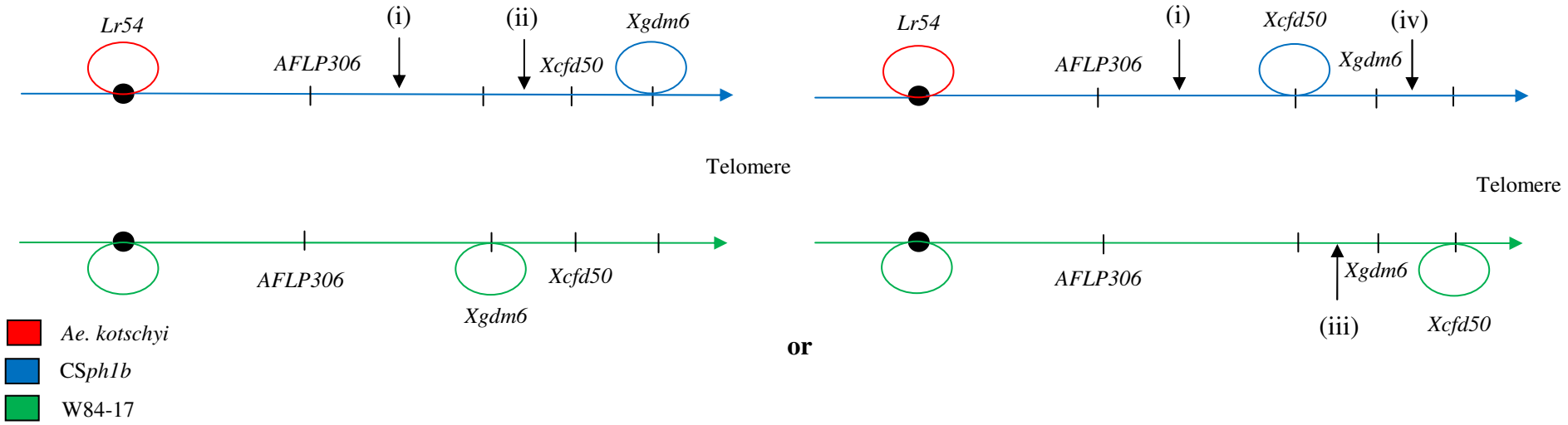



Figure 3.12 Second (homologous) meiotic recombination event involving chromosome arm 2DL of W84-17 and a recombined *Ae. kotschyi*/CS 2DL chromosome arm. An internal chromosomal translocation involving *Xgdm6* and *Xcfd50* may have resulted in a different locus order between CS*ph1b* and W84-17. Meiotic recombination in either of these pairing structures would have resulted in normal gametes as well as gametes containing deletions (*Xgdm6*) and duplications (*Xcfd50*). The arrows indicate places where single crossovers could have occurred in the formation of the different recombinants.

carries null alleles for the respective loci, which can therefore be used for the identification of translocation homozygotes. However, in order for them to be employed effectively in this manner, it will be necessary to determine beforehand whether the normal wheat polymorphisms also include null alleles. Null alleles are often one of the polymorphisms seen at a microsatellite locus and this will create a risk for misidentification, thus detracting from the value of the marker. However, by evaluating the three loci simultaneously, the accuracy of marker-aided identification can be improved considerably. Lines homozygous for the translocation will therefore have the null allele at all three marker loci, a situation that will only occur in a very low percentage of normal wheat lines. If the three microsatellite amplification reactions can be multiplexed it will further add to the usefulness of the assay.

The possibility of simultaneously testing for the absence of the three microsatellite marker loci, *Xbarc228*, *Xcfd233* and *Xwmc41*, by employing a multiplex PCR reaction was investigated. First of all it was necessary to confirm that the three loci can be scored accurately by separation of the PCR product on 2% agarose gels and visualizing it with the use of ethidium bromide under UV light rather than separating on acrylamide and visualizing with silver stain. Scoring on agarose gels will simplify the screening of large number of plants in MAS. The three primer sets were used to screen the genotype panel *Ae. kotschyi*, CS-S14 (hemi), rec. #74, CS and W84-17. The results are shown in Fig. 3.13.

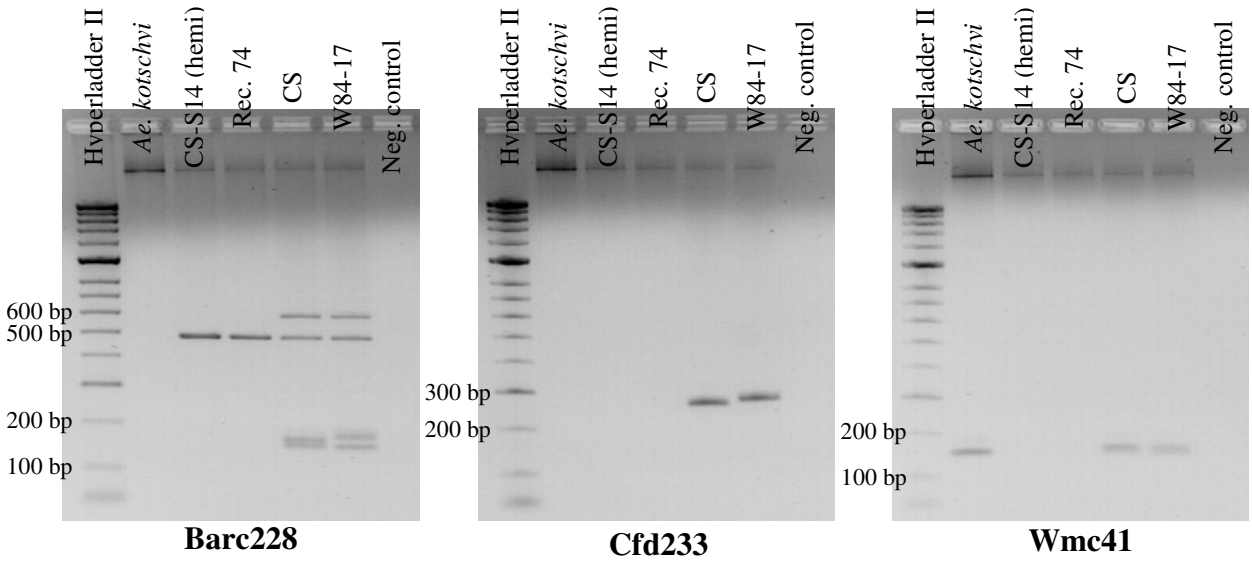



Figure. 3.13 Detection of microsatellite loci *Xbarc228*, *Xcfd233* and *Xwmc41* using 2% agarose gels and visualization under UV light.

Marker Barc228 amplified two bands, 600 bp and 140 bp, respectively that occur only in CS and W84-17. In addition this marker also amplified a 150 bp band in W84-17 and a 500 bp band that occurred in all the controls except, *Ae. kotschyi* (and which probably does not derive from 2DL). Marker Cfd233 similarly amplified 290 bp and 300 bp bands in CS and W84-17, respectively. Marker Wmc41 amplified a 170 bp band in CS, W84-17 and *Ae. kotschyi* which was absent in CS-S14 (hemi). The presence of a band in *Ae. kotschyi* but not in CS-S14 (hemi) may be the result of it being a tetraploid (genomes UUSS); with only one of its group 2 chromosomes amplifying a band. Secondly, it is possible that the band amplified in *Ae. kotschyi* is not homoeoallelic to the wheat bands from 2DL.

Thus, the results show that all three loci can be used as recessive markers employing a rapid and simple PCR and visualization protocol. If all three loci are scored simultaneously in a haplotype assay, the chance of misidentification through the occurrence of null alleles in the wheat breeding population becomes very small. As a result the screening procedure will be highly specific. If the three analyses can be multiplexed it will further simplify the test.

The PCR reactions with Cfd233 and Wmc41 were multiplexed to increase the cost efficiency of detecting recombinant #74. Marker Barc288 was not included in the multiplex PCR reaction of Fig. 3.14 since the resolution of 2% agarose gel was insufficient to distinguish between the 140 bp *Xbarc228* allele and the 170 bp *Xwmc41* allele. However, this would not matter in marker assisted selection as the null condition will be scored.

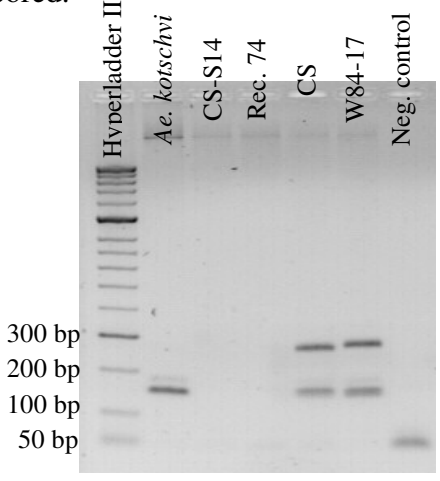



Figure 3.14 Simultaneous detection of microsatellite loci *Xcfd233* and *Xwmc41* in a multiplexed PCR reaction of which the amplification products were separated on a 2% agarose gel and visualized under UV light.

## 3.3 Identification of additional molecular markers that can be used for the development of a dominant SCAR marker for the detection of recombinant #74 and/or the further characterization of the recombinants.

### 3.3.1 RAPDs

Twelve RAPD primers that were polymorphic for CS-S14 but had low reproducibility were identified by Eksteen (2008). These were re-evaluated in an attempt to find polymorphic fragments associated with the translocation recombinants. For this purpose the primer sets were tested on DNA derived from a plant panel consisting of *Ae. kotschyi*, CS-S14 (hemi), rec. #74, CS and W84-17. The RAPD profiles obtained are shown in Fig. 3.15 and a summary of the polymorphisms observed are given in Table 3.4.

Table 3.4 A summary of the polymorphisms observed (Fig. 3.15) when 12 RAPD primers were tested on a plant panel consisting of *Ae. kotschyi*, CS-S14 (hemi), rec. #74, CS and W84-17.

| Primer | CS vs. W84-17 | CS and W84-17 vs. CS-S14 (hemi) |
|---|---|---|
| OPE19 | 1 | 1 |
| OPF16 | 3 | 0 |
| OPN8 | 0 | 0 |
| OPN12 | Did not amplify in CS | Did not amplify in CS |
| OPN13 | 3 | 0 |
| OPN20 | 2 | 0 |
| OPP4 | 1 | 0 |
| OPQ7 | 0 | 0 |
| OPR4 | 1 | 0 |
| OPT5 | 2 | 0 |
| OPU9 | 2 | 0 |
| OPU11 | 1 | 0 |

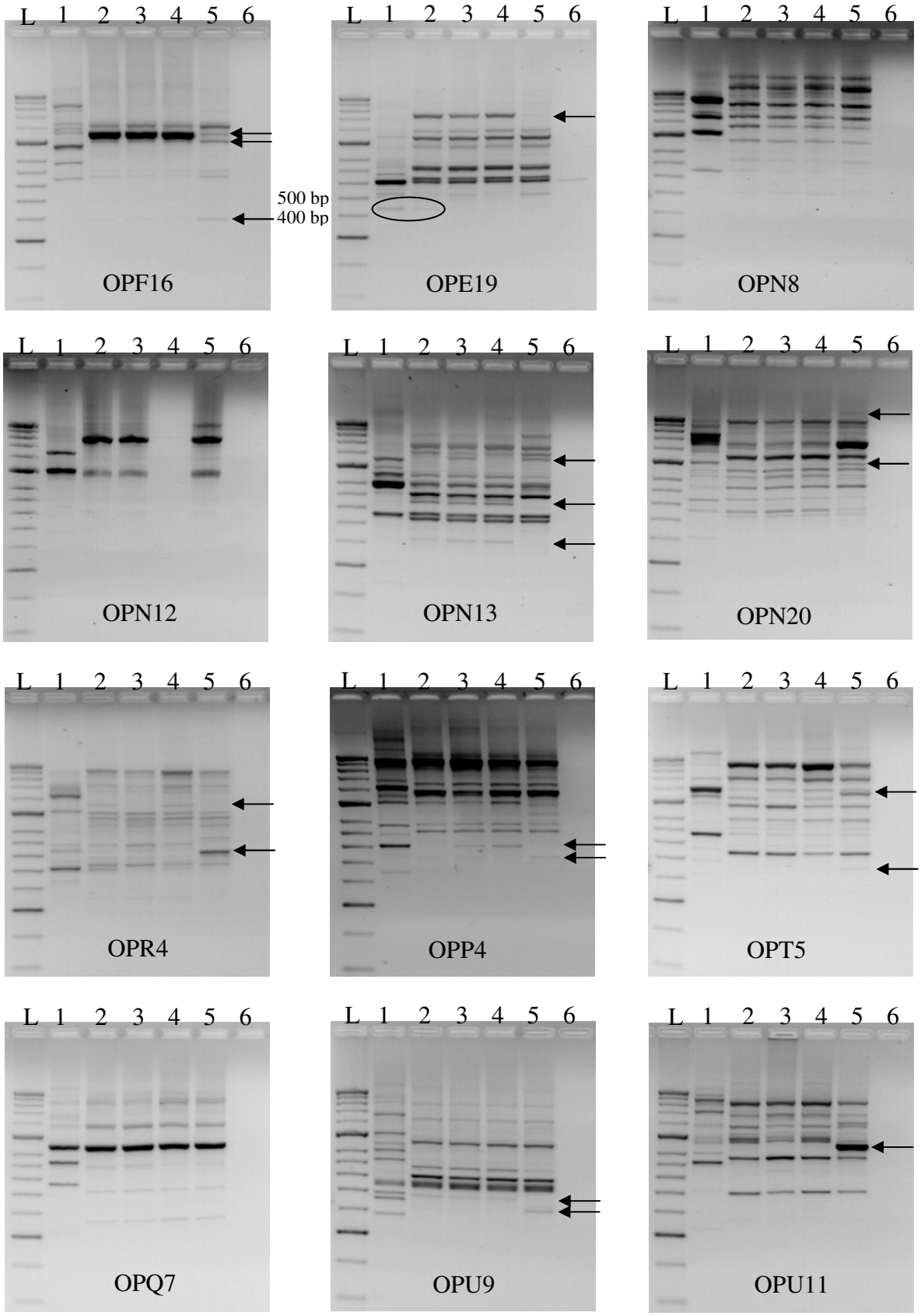



Figure 3.15 A summary of the data obtained with 12 RAPD primers that were re-evaluated to determine if they are polymorphic for the shortest recombined translocation. The control panel used to test these primers was as follow: (L) HyperLadder II (1) *Ae. kotschyi*, (2) CS-S14 (hemi), (3) Rec. #74, (4) CS, (5) W84-17 and (6) Negative control. The arrows indicate polymorphisms between CS and W84-17 whereas the circle indicates a polymorphism specific for the S14 translocation.

The primary objective was to identify RAPDs that amplify a band in *Ae. kotschyi*, CS-S14 (hemi) and rec. #74 but not in CS and W84-17. No such polymorphism was detected. However, RAPD primer OPE19 amplified a band in *Ae. kotschyi* and CS-S14 (hemi) that was absent in rec. #74, CS and W84-17 suggesting that this polymorphism were specific for the S14 translocation, and that the corresponding locus map distally from the position on 2DL where the most distal allosyndetic crossover occurred (Table 3.3). Eksteen (2008) evaluated 230 RAPD primers on a test panel that included CS-S14 (hemi) and rec. #74 to identify the 12 putative polymorphisms for CS-S14 (hemi). As a result further evaluation of RAPD primers was considered unlikely to produce the desired polymorphisms.

### 3.3.2 RGAs

Nine degenerate primers based on the conserved amino acid motifs of resistance genes in tomato, wheat and rice and derived by Shi *et al*. (2001) and Yan *et al*. (2003) were used in a further attempt to find unique sequences associated with the *Ae. kotschyi* chromatin in the recombinants. The primers were used in all possible combinations of two for a total of 36 combinations. These primer combinations were tested on a genotype panel consisting of *Ae. kotschyi*, CS-S14 (hemi), rec. #74, CS and W84-17. The PCR products were size separated on a 6% denaturing poly-acrylamide sequencing gel and visualized with silver staining. Each primer combination was analyzed by scoring bands present in CS-S14 (hemi) and absent in CS and W84-17. The number of polymorphisms detected for each primer combination is summarized in Table 3.5.

All of the nine RGA primers detected loci that were polymorphic between CS-S14 (hemi) and CS/W84-17 when used in pairs. In total the primer combinations detected 87 such polymorphisms. However, three RGA primers, Ptokin1-IN, Cre3LR-R and Xa1LR-F, were found to generate the most polymorphisms and detected 26, 29 and 35 polymorphic loci, respectively. Since the primary objective was to find polymorphisms that map to the shortest translocation, polymorphic loci that occur in both CS-S14 and the shortest recombinant were singled out. Five of the 87 loci also occurred on the shortest recombinant. These were produced by the following primer

combinations: Ptokin1-IN/Cre3LR-R; Ptokin1-IN/Xa1LR-F; Ptokin2-IN/Cre3LR-R; Cre3LR-R/Xa1LR-F and Cre3LR-R/Xa1NBS-R (Fig. 3.16).

Table 3.5 The RGA primer combinations used in conjunction with a panel of five genotypes (*Ae. kotschyi*, CS-S14 (hemi), rec. #74, CS and W84-17) and the number of polymorphisms detected per primer combination between the complete translocation, CS-S14 (hemi) and both CS and W84-17.

| | **Ptokin1 IN** | **Ptokin2 IN** | **Cre3LR R** | **Xa1LR F** | **Xa1LR R** | **Xa1NBS F** | **Xa1NBS R** | **CLRR INV1** | **XLRR INV2** |
|---|---|---|---|---|---|---|---|---|---|
| **Ptokin1-IN** | | 2 | 3 | 12 | 1 | 1 | 4 | 2 | 1 |
| **Ptokin2-IN** | | | 5 | 5 | 1 | 1 | 2 | 0 | 3 |
| **Cre3LR-R** | | | | 6 | 2 | 1 | 9 | 2 | 1 |
| **Xa1LR-F** | | | | | 6 | 0 | 4 | 2 | 0 |
| **Xa1LR-R** | | | | | | 2 | 3 | 0 | 1 |
| **Xa1NBS-F** | | | | | | | 1 | 1 | 2 |
| **Xa1NBS-R** | | | | | | | | 0 | 0 |
| **CLRR-INV1** | | | | | | | | | 1 |
| **XLRR-INV2** | | | | | | | | | |

The majority of polymorphic loci were low intensity bands and the PCR reactions of the five primer pairs that seemingly detected polymorphic loci which were specific for the shortest translocation were retested on the same genotype panel. None of the polymorphisms could be confirmed (Fig. 3.16). Some of the bands did not amplify at all in the second amplification while in the other cases a similar band was amplified in the controls, CS and/or W84-17. In the attempt to confirm the polymorphisms it was noticed that the pronounced bands in an RGA profile were more re-producible than the faint bands. Unfortunately, all the polymorphisms that were detected (Table 3.5) were faint bands. However, only a small number of polymorphic loci were re-evaluated and it does not necessarily mean that all the remaining putative polymorphisms in Table 3.5 will also be non-reproducible. Yet, due to the difficulty of accurately scoring the faint bands, it was decided not to use the remaining putative RGA polymorphisms for the characterization of the full set of recombinants.

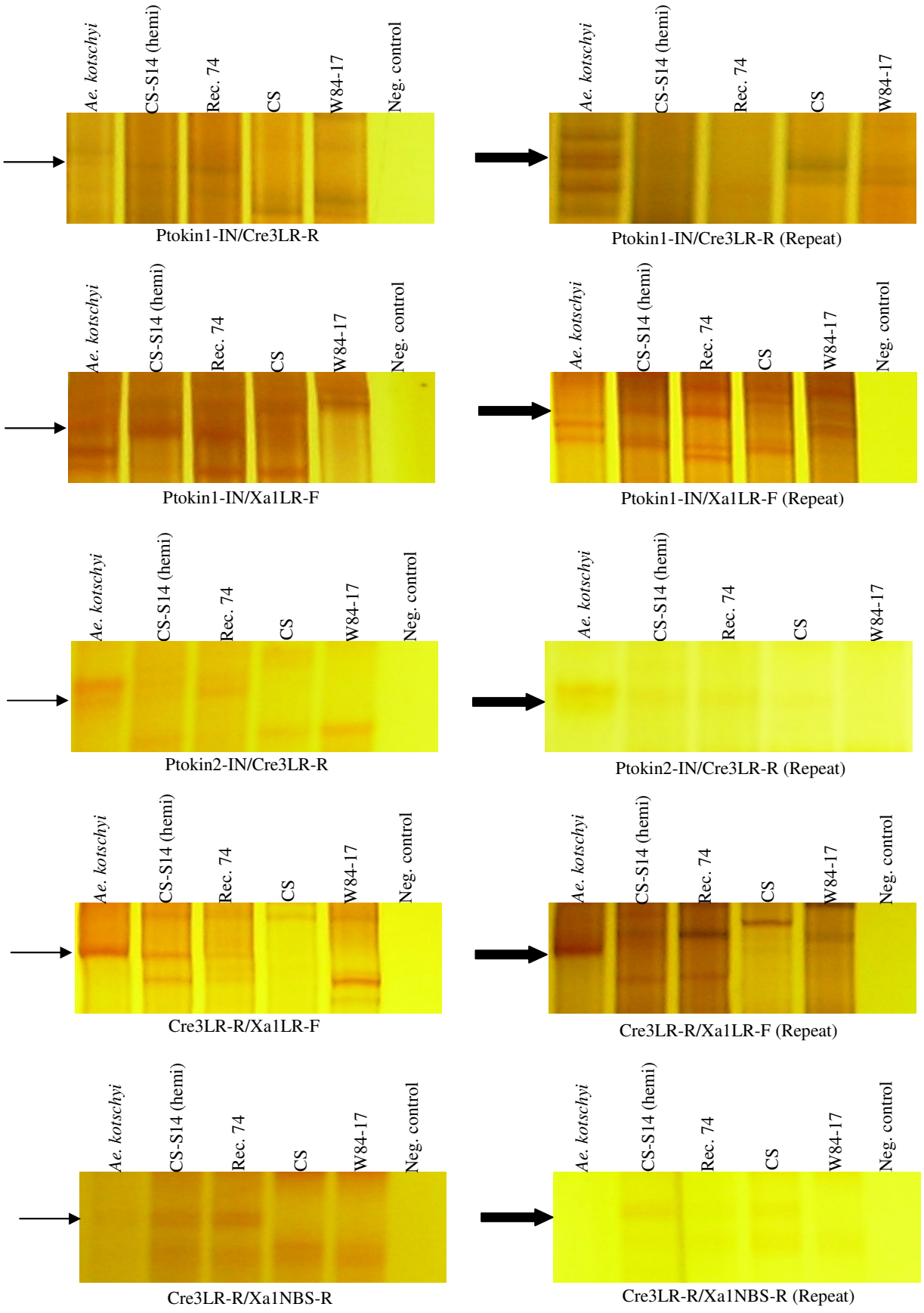



Figure 3.16 Polymorphic loci for the shortest recombined translocation that were detect with the aid of five resistance gene analog (RGA) primer combinations. The PCR reactions were then repeated to confirm the reproducibility of the diagnostic bands. Arrows indicate the polymorphisms amplified in the first PCR reactions whereas block arrows indicate the polymorphisms seen when PCR reactions were repeated.

### 3.3.3 AFLPs

AFLP markers were evaluated on a genotype panel consisting of *Ae. kotschyi*, CS-S14 (hemi), CS, W84-17 and the 10 recombinants in an attempt to identify polymorphic loci that map to the translocation. Fluorescently labeled *Eco*RI primers were used in combination with five unlabeled *Mse*I primers with three selective base pairs (CAG, CTG, CAT, CTC, CTA) in the selective amplification step. The PCR products were separated with an automated sequencer and analyzed using Genemapper® softwear IV. A locus was scored as polymorphic when a band was present in CS-S14 (hemi) but absent in CS and W84-17 or when a band was present in CS and W84-17 but absent in CS-S14 (hemi).

A total of 15 putative polymorphic loci were amplified by four selective primers, CAG, CTG, CAT and CTA whereas selective primer CTC did not amplify any polymorphic loci (Table 3.6; Addendum D). To confirm the repeatability of the polymorphisms and its association with the S14 translocation, AFLP analysis was repeated with the respective primer combinations. The second set of analyses were conducted on a panel of 16 plants, eight with (resistant) and eight without (susceptible) the S14 translocation (S14 translocation 2*/CS*ph1b* mutant). The Genemapper® profiles that were obtained are given in Addendum E and the data are summarized in Table 3.7.

Six polymorphisms (produced by *Eco*RI – AGG/*Mse*I – CAG 212, *Eco*RI – AGG/*Mse*I – CAG 228, *Eco*RI – AAC/*Mse*I – CAT 410, *Eco*RI – ACA/*Mse*I – CTG 306, *Eco*RI – AGG/*Mse*I – CTG 360, *Eco*RI – AAC/*Mse*I – CTG 416) were both repeatable and co-segregated with the S14 translocation. However, of these polymorphisms only the *Eco*RI – AAC/*Mse*I – CAT 410 product was specific for the shortest recombinant (rec. #74).

Since rec. #74 was identified as the shortest recombinant with only the proximal region consisting of foreign chromatin (Fig. 3.9), it was expected that *Eco*RI – AAC/*Mse*I – CAT 410 would produce the *Ae. kotschyi* polymorphism in all the recombinants. However, following the first AFLP analysis (Table 3.6) recombinants

Table 3.6 A summary of polymorphic AFLP loci detected in *Ae. kotschyi*, CS-S14 (hemi), CS, W84-17 and the 10 recombinants using labeled *Eco*RI primers (ACA, AAC, AGG) in combination with *Mse*I selective primers CAG, CTG, CAT, CTC and CTA.

| **Primer comb.** | **Allele (bp)** | ***Ae. kotschyi*** | **CS-S14** | **CS** | **W84-17** | **25** | **37** | **74** | **119** | **148** | **205** | **247** | **256** | **265** | **273** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Eco*RI - ACA *Mse*I – CAG | 314 | W | S | W | W | S | S | W | W | S | S | W | S | W | W |
| *Eco*RI - AGG *Mse*I - CAG | 212 | S | S | W | W | S | W | W | S | S | W | S | S | S | S |
| | 228 | S | S | W | W | S | W | W | S | S | W | S | S | S | S |
| *Eco*RI - ACA *Mse*I - CAT | 78 | S | S | W | W | S | S | W | S | S | S | S | S | S | S |
| | 212 | S | S | W | W | S | S | W | S | S | S | S | S | S | S |
| *Eco*RI - AAC *Mse*I - CAT | 238 | W | S | W | W | S | S | S | W | W | W | S | W | S | S |
| | 410 | S | S | W | W | S | S | S | S | W? | S | W? | W? | S | S |
| *Eco*RI - ACA *Mse*I - CTA | 273 | S | S | W | W | S | S | W | S | S | S | S | S | S | S |
| | 315 | S | S | W | W | S | S | W | S | S | S | S | S | S | S |
| | 378 | S | S | W | W | S | W | W | S | S | W | S | S | S | S |
| *Eco*RI - AGG *MseI* - CTA | 73 | W | S | W | W | S | S | W | S | S | S | S | S | S | S |
| *Eco*RI - ACA *Mse*I - CTG | 231 | S | S | W | W | W | W | S | S | W | S | W | W | W | S |
| | 306 | S | S | W | W | S | S | W | S | S | S | S | S | S | S |
| *Eco*RI - AGG *Mse*I – CTG | 360 | S | S | W | W | S | S | W | S | S | S | S | S | S | S |
| *Eco*RI - AAC *Mse*I - CTG | 416 | S | S | W | W | S | S | W | S | S | S | S | S | S | S |

W – Wheat polymorphism
S – *Ae. kotschyi* polymorphism

Table 3.7 Results obtained following confirmation screening of plants with (resistant) and without (susceptible) the S14 translocation using polymorphic AFLP *Mse*I selective primers.

| Primer comb. | Allele (bp) | CS | CS-S14 | R1 | R2 | R3 | R4 | R5 | R6 | R7 | R8 | S1 | S2 | S3 | S4 | S5 | S6 | S7 | S9 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Eco*RI - ACA *Mse*I – CAG | 314 | W | S | W | W | W | W | W | W | W | W | W | W | W | W | W | W | W | W |
| *Eco*RI - AGG *Mse*I - CAG | 212 | W | S | S | S | S | S | S | S | S | S | W | W | W | W | W | W | W | W |
| | 228 | W | S | S | S | S | S | S | S | S | S | W | W | W | W | W | W | W | W |
| *Eco*RI - ACA *Mse*I - CAT | 78 | W | S | W | W | W | W | W | W | W | W | W | W | W | W | W | W | NA | W |
| | 212 | W | S | W | W | W | W | W | W | W | W | W | W | W | W | W | W | NA | W |
| *Eco*RI - AAC *Mse*I - CAT | 238 | W | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | NA | S |
| | 410 | W | S | S | S | S | S | S | S | S | S | W | W | W | W | W | W | NA | W |
| *Eco*RI - ACA *Mse*I - CTA | 273 | W | S | W | W | W | W | W | W | W | W | W | W | W | W | W | W | W | W |
| | 315 | W | S | W | W | W | W | W | W | W | W | W | W | W | W | W | W | W | W |
| | 378 | W | S | W | W | W | W | W | W | W | W | W | W | W | W | W | W | W | W |
| *Eco*RI - AGG *Mse*I - CTA | 73 | W | S | W | W | W | W | W | W | W | W | W | W | W | W | W | W | W | W |
| *Eco*RI - ACA *Mse*I - CTG | 231 | W | S | W | S | S | S | W | W | S | S | W | W | W | S | W | S | W | W |
| | 306 | W | S | S | S | S | S | S | S | S | S | W | W | W | W | W | W | W | W |
| *Eco*RI - AGG *Mse*I – CTG | 360 | W | S | S | S | S | S | S | S | S | S | W | W | W | W | W | W | W | W |
| *Eco*RI - AAC *Mse*I - CTG | 416 | W | S | S | S | S | S | S | S | S | S | W | W | W | W | W | W | W | W |

W – Wheat polymorphism
S – *Ae. kotschyi* polymorphism
NA – No amplification

#148, #247 and #256 seemed to produce the corresponding wheat polymorphism. It was therefore necessary to confirm whether the *Eco*RI – AAC/*Mse*I – CAT 410 amplification product was in fact associated with the shortest recombinant. For this purpose resistant and susceptible $BCF_2$ progeny of rec. #74 (cross = CS-S14 translocation/2*CS*ph1b* mutant//W84-17/3/CSN2DT2A-B/4/2*W84-17) were tested for the presence of the *Eco*RI – AAC/*Mse*I – CAT 410 band. The locus proved to be specific for rec. #74 when it only amplified in resistant plants (Addendum F). An attempt was therefore made (section 3.3.5) to develop a SCAR marker from the *Eco*RI – AAC/*Mse*I – CAT 410 fragment.

### 3.3.4 Evaluation of the recombinants with existing SCAR markers

A SCAR marker, Ust2-$IIJ_1^d$, which was developed for chromosome $2J_1^d$ of *Thinopyrum distichum*, amplifies a 597 bp band in *Th. distichum* (Marais *et al*., 2007). However, this marker also detects a locus on chromosome arm 2DL of wheat and Marais *et al*. (2009) recently developed an improved marker specific for chromosome 2D. Marker Ust2-$IIJ_1^d$ was tested against *Ae. kotschyi,* CS-S14 (hemi), CS, W84-17 and the 10 recombinants in an attempt to determine its approximate location on 2DL (Fig. 3.17).

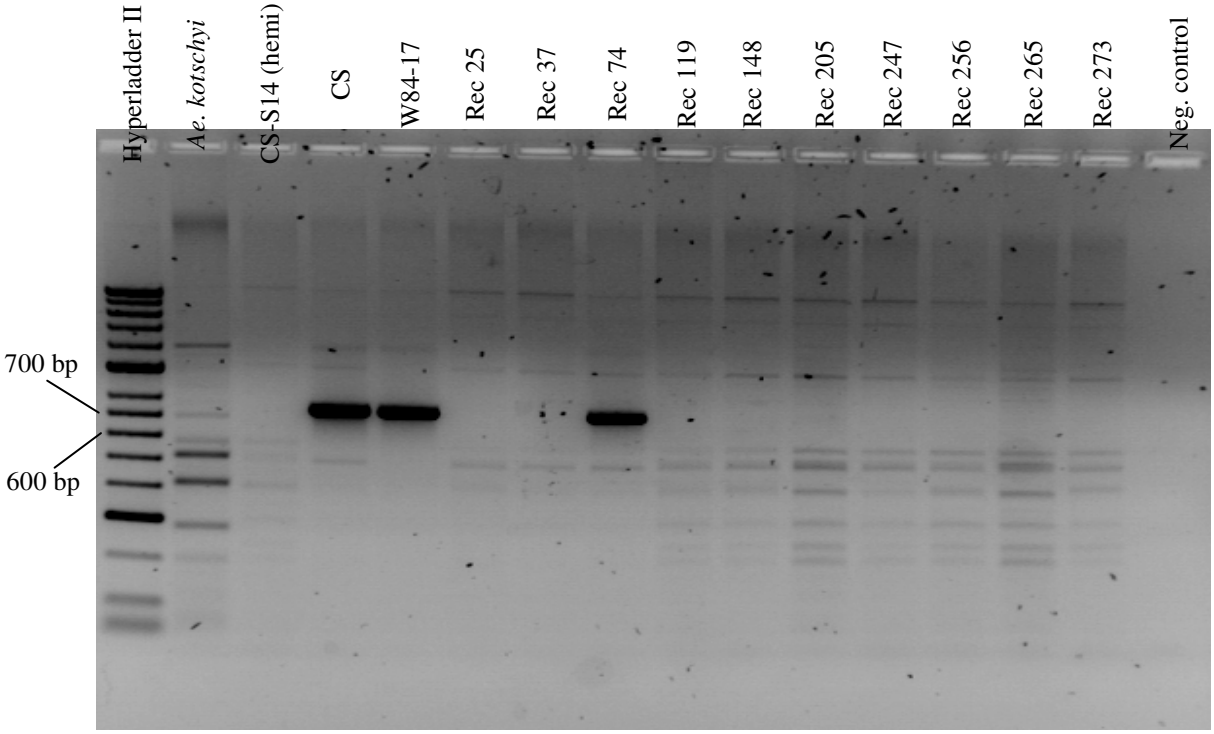



Figure 3.17 Results obtained when the 10 S14 recombinants and control genotypes were tested for the presence of the SCAR locus *Xust2-$IIJ_1^d$*.

The SCAR marker amplified a single prominent band (approx. 700 bp) in CS and W84-17 but not in CS-S14 (hemi). The same band was also amplified in rec. #74 but was absent in the remaining recombinants. The additional molecular marker data (AFLP, SCAR) obtained for the 10 recombined translocations were integrated with the microsatellite data provided in Table 3.3 and are presented in Table 3.8. The data suggest that *Xust2-IIJ*$_1^d$ occurs outside of the region spanned by the shortest translocation (rec. #74) and that it is situated between the rec. #74 translocation breakpoint and AFLP marker 228 on the translocation.

In addition Eksteen (2008) developed a SCAR marker, Sopw7, for an *Ae. neglecta* derived translocation in wheat. The SCAR primers also amplify similar size fragments in other wild species derived translocations (Marais 2008; personal communication) and were therefore tested against *Ae. kotschyi*, CS-S14 (hemi), CS, W84-17 and the 10 translocation recombinants (Fig. 3.18).

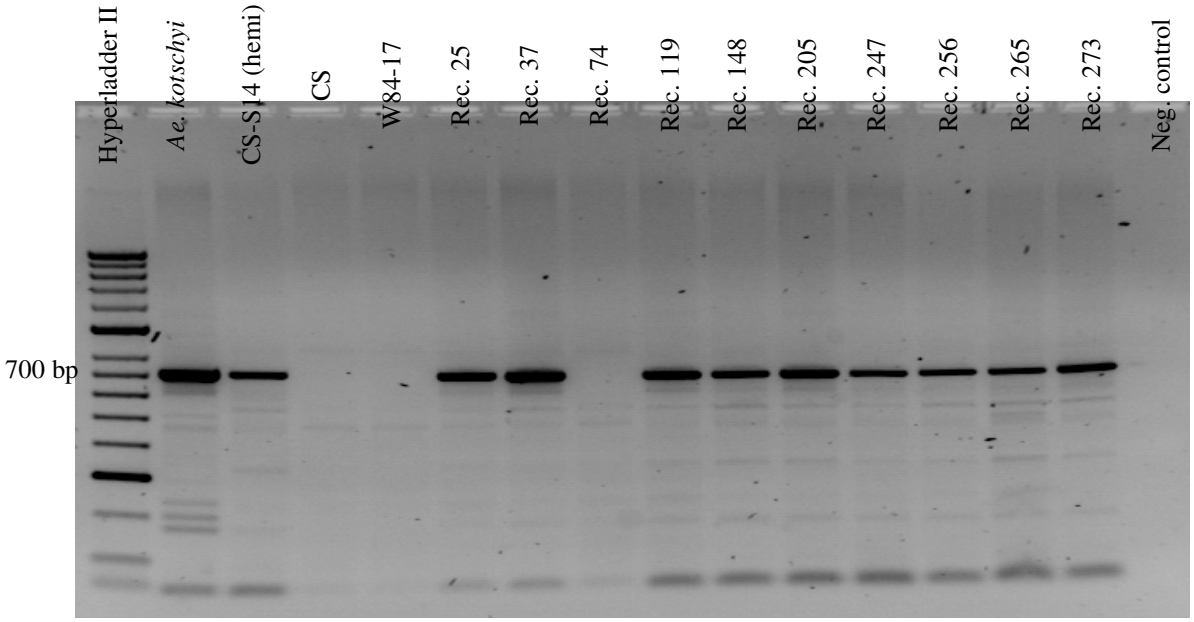



Figure 3.18 Results obtained following screening of the 10 S14 recombinants and control genotypes with SCAR marker Sopw7.

SCAR marker Sopw7 amplified a 700 bp band in *Ae. kotschyi* and CS-S14 (hemi) that was absent in CS and W84-17. The same band was also amplified in nine of the recombinants but was absent in rec. #74. SCAR marker Sopw7 is therefore not specific for the shortest recombinant and its locus occurs between microsatellite marker Gwm539 and AFLP marker 228 (Table 3.8).

### 3.3.5 Conversion of a polymorphic AFLP locus into a SCAR marker

AFLP analysis of control lines and 10 recombinants revealed six polymorphic loci specific for the S14 translocation. However, only one locus, *Eco*RI – AAC/*Mse*I – CAT 410, proved to be specific for the shortest recombinant (rec. #74) and an attempt was made to convert this locus into a SCAR marker. Since AFLP analysis was initially performed using fluorescence labeled primers, AFLP analysis was repeated using unlabeled *Eco*RI – AAC and *Mse*I – CAT primers and the product was separated on a 6% (w/v) denaturing poly-acrylamide sequencing gel and silver stained in order to recover the 410 bp fragment. Since labeled and unlabeled bands were visualized by silver staining, the 410 bp fragment was not visible as a single polymorphic entity and the area surrounding the 410 bp band was excised from the *Ae. kotschyi* and CS (negative control) lanes and the DNA recovered (Fig. 3.19). The DNA was re-amplified with unlabeled *Eco*RI – AAC and *Mse*I – CAT primers and the product separated on a 2% agarose gel. However, no distinctive polymorphic 410 bp band was visible in *Ae. kotschyi* and the area surrounding 410 bp was therefore re-excised, the DNA recovered and cloned into a pGem – T Easy vector system.

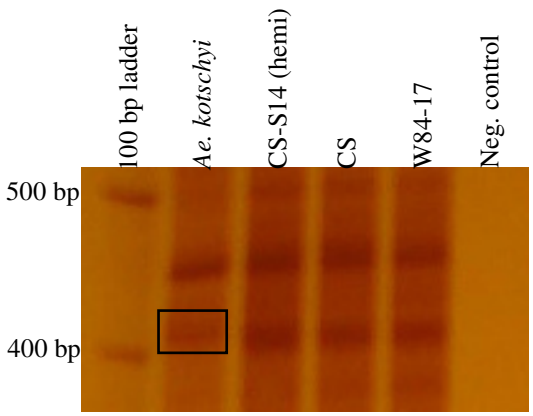



Figure 3.19 Separation of DNA fragments on a 6% (w/v) denaturing poly-acrylamide sequencing gel following amplification with the unlabeled AFLP primers *Eco*RI – AAC and *Mse*I – CAT. The rectangle demarcates the area containing the 410 bp polymorphic fragment that was excised from *Ae. kotschyi*.

Screening of clones using the T7 and Sp6 vector primers revealed clones with different insert sizes. However, the resolution of agarose gels was insufficient to distinguish between the clone inserts that varied from each other with respect to a few base pairs only. Clones that seemed to have the correct insert size were therefore

sequenced in order to determine their precise insert size. Following the screening and sequencing of various clones, a clone with an insert size of 411 bp was identified and primers were designed using the program OligoAnalyzer (Fig. 3.20).

5' GACTGCGTACCAATTCAACTTGCCAAGAGGAGGTGGTTTCACCATATTCCC
ATGCCAACCACGTCTATTGCTGCAATATATCCGTCCTCTGAGTAGGAGGTA
AGTGGTCAGGCGTTGTCTGGCCAGACAAATCTGACCATGCGCTAGTTTTGT
GCCCATTCACATTTTGCAGATGCTCATCCAGGGTCTGCTAGTAGGTGTCTC
CGGATTTTTGGAGCCCAAATTACCGTCATGGGCCGAAGCTCGACCGCGAA
CATGTCGCGAGGGTTGGTGAGTCCACATACACGATCTGGCAAATCGGTGC
GAGGCACATACGGAAGTCACCATGGTTGTTGATGATGAAGGCAACGGCAC
CCTCATGGAGCGGGCACACAAGGTGTGTCGTTTTCTGCATGTTACTCAGGA
CTCATC 3'

Figure 3.20 Forward sequence of the *Eco*RI – AAC/*Mse*I – CAT fragment. The Eco*RI* and Mse*I* primers are indicated in red.

The primers (forward primer: 5' ACCAATTCAACTTGCCAAGAG 3'; reverse primer: 5' GAGTAACATGCAGAAAACGACA 3') were evaluated on the 10 recombinant lines (Fig. 3.21) as well as on panels of resistant and susceptible $BCF_2$ and $BCF_3$ progeny segregating for the presence of rec. #74 (Addenda G and H). Also included were the parental lines *Ae. kotschyi*, CS-S14 (hemi), CS and W84-17.

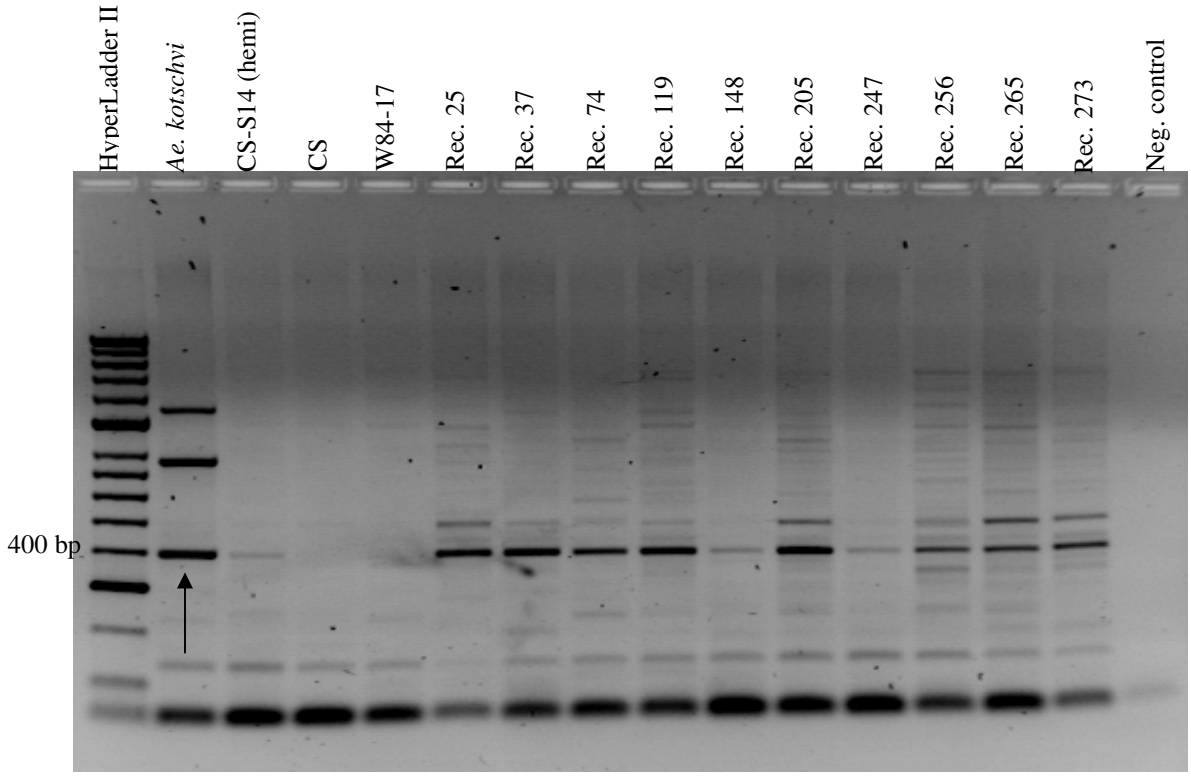



Figure 3.21 Evaluation of newly designed primers that target a 410 bp polymorphic AFLP band in 10 S14 recombinants and control genotypes.

The new primer set (*Xscar410*) developed for the 410 bp polymorphic AFLP fragment amplified a 410 bp band in *Ae. kotschyi* and CS-S14 (hemi) that was absent in CS and W84-17. The marker proved to be specific for the shortest translocation recombinant as it amplified a clear band in resistant $BCF_2$ (Addendum G) and $BCF_3$ (Addendum H) plants. A faint band that appears to be slightly smaller than the 410 bp band was amplified in some susceptible $BCF_2$ and $BCF_3$ (S5, S9) plants.

### 3.4 Characterization of the S14 translocation and recombinants with chromosome 2DS microsatellite loci

In this study microsatellite, AFLP and SCAR markers primarily located on chromosome arm 2DL were used to identify and characterize translocation recombinants (Table 3.8). In addition to these, four microsatellite markers specific for chromosome arm 2DS were used to confirm that the translocation is probably confined to chromosome arm 2DL as was suggested by Marais *et al*. (2005). Markers Gwm484, Gwm261, Cfd116 and Barc124 were evaluated on a test panel (Table 2.1) and used to screen the 10 putative recombinants (Addendum C). Since marker Gwm261 is diagnostic for *Rht8* its inclusion would confirm the presence of wheat chromatin at the *Rht8* location on chromosome arm 2DS.

The four markers were confirmed to be specific for chromosome arm 2DS (Addendum C). Each amplified fragments in *Ae. kotschyi*, yet these were clearly not associated with the translocated region that carried the rust resistance genes. Marker Cfd116 amplified the wheat allele in all the recombinants, whereas markers Gwm484, Gwm261 and Barc124 amplified the wheat allele in nine of the translocation recombinants and a null allele in rec. #74. Thus, the translocation does not extend into the chromosome arm 2DS region where these loci occurred.

Markers Gwm484, Gwm261 and Cfd116 were mapped in the most proximal deletion bin on chromosome arm 2DS (Sourdille *et al*., 2004). Since recombination is partially suppressed in areas surrounding the centromere (Werner *et al*., 1992; Delaney *et al*., 1995a, b; Michelson-Young *et al*., 1995), the relative locations of the three loci could be deduced from the data of Table 3.8. In the first (allosyndetic) meiosis (Fig. 3.11), translocation of the alien segment onto a CS chromosome 2D was

Table 3.8 A summary of all the marker data used to identify and characterize the translocation recombinants.

| Line | SSR *Xgwm261* (2DS) | SSR *Xgwm484* (2DS) | SSR *Xbarc124* (2DS) | SSR *Xcfd116* (2DS) | SSR *Xbarc228* *Xwmc41* *Xcfd233* *Xscar410* *Lr54* *Yr37* | SSR *Xgwm157* *Xgwm539* *(H)* | AFLP -306 -360 -416 *Xust2-IIJ$_1$$^d$* *Xsopw7* | AFLP -228 -212 | SSR *Xcfd50* | SSR *Xwmc167* | SSR *Xgdm6* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Ae. kotschyi*** | | | | | S | S | S | S | S* | S | S |
| ***Lr54/Yr37*** **hemizygote** | W | W | W | $W^{CS}$ | S | S | S | S | S | S | S |
| **CS** | $W^{CS}$ | $W^{CS}$ | $W^{CS}$ | $W^{CS}$ | W | W | W | W | $W^{CS}$ | W | $W^{CS}$ |
| **W84-17** | $W^{W}$ | $W^{W}$ | $W^{W}$ | $W^{W}$ | W | W | W | W | $W^{W}$ | W | $W^{W}$ |
| **Rec. 74** | - | - | - | $W^{CS}$ | S | $W^{CS}$ | W | W | $W^{CS}$ & $W$ | W | $W^{W}$ |
| **Rec. 37** | $W^{W}$ | $W^{W}$ | $W^{W}$ | $W^{CS}$ | S | S | S | W | $W^{W}$ | W | $W^{W}$ |
| **Rec. 205** | $W^{CS}$ | $W^{CS}$ | $W^{CS}$ | $W^{CS}$ | S | S | S | W | $W^{W}$ | W | - |
| **Rec. 256** | $W^{W}$ | $W^{W}$ | $W^{W}$ | $W^{CS}$ | S | S | S | S | $W^{CS}$ | W | $W^{CS}$ |
| **Rec. 119** | $W^{W}$ | $W^{W}$ | $W^{CS}$ | $W^{CS}$ | S | S | S | S | $W^{W}$ | W | - |
| **Rec. 148** | $W^{W}$ | $W^{CS}$ | $W^{CS}$ | $W^{CS}$ | S | S | S | S | $W^{W}$ | W | - |
| **Rec. 247** | $W^{W}$ | $W^{CS}$ | $W^{CS}$ | $W^{CS}$ | S | S | S | S | $W^{W}$ | W | - |
| **Rec. 265** | $W^{CS}$ | $W^{CS}$ | $W^{CS}$ | $W^{CS}$ | S | S | S | S | $W^{CS}$ & $W$ | W | $W^{CS}$ |
| **Rec. 273** | $W^{CS}$ | $W^{CS}$ | $W^{W}$ | $W^{CS}$ | S | S | S | S | $W^{W}$ | W | - |
| **Rec. 25** | $W^{W}$ | $W^{CS}$ | $W^{CS}$ | $W^{CS}$ | S | S | S | S | $W^{W}$ | W | - |

Symbols used for the data: "-" indicate a deleted region; "$W^{W}$" indicate the W84-17 allele; "$W^{CS}$" indicate the CS allele, "W" indicate a wheat allele; "S" indicate the *Ae. kotschyi* allele whereas "S*" indicates the amplification of fragments in *Ae. kotschyi* that are unrelated to the S14 translocated segment.

achieved. In the second (homologous) meiosis (Fig. 3.12) the CS-S14 translocation chromosome recombined with chromosome 2D of W84-17. It is likely that the resistance-carrying recombined chromosomes that were subsequently selected would have retained regions of CS chromatin close to the centromeres with W84-17 chromatin occurring towards the telomeres. The areas consisting of W84-17 chromatin will have occurred more frequently towards the telomere as the frequency of recombination increases towards the telomere (Werner *et al*., 1992; Delaney *et al*., 1995a, b; Michelson-Young *et al*., 1995). Since marker Cfd116 amplified the CS allele in all the translocation recombinants, this marker was regarded as the most proximal marker on chromosome arm 2DS followed by markers Barc124, Gwm484 and Gwm261. The map position of *Xbarc124* as suggested by the present data is inconsistent with its previously mapped location in the most distal deletion bin on 2DS (Sourdille *et al*., 2004). Instead marker Barc124 appears to be located in-between *Xgwm484* and *Xcfd116*, both of which were mapped to the most proximal deletion bin by Sourdille *et al*. (2004). This difference could be the result of chromosomal variation between the wheat mapping populations used or the possibility that the present result or the published marker order is incorrect.

The 2DS microsatellite data of Table 3.8 suggests that the rec. #74 chromosome 2D contains a large deletion on its short arm. Markers Gwm261, Gwm484 and Barc124 were lost in rec. #74 and it appears to have retained only the centromere as well as a proximal part of 2DS. The rest of the arm was affected by a deletion that could either extend up to and including the telomere or could involve a large intercalary region.

Depending on the size of the deletion and the genes (for example *Rht8*) that it includes, the deletion may affect phenotypic characteristics such as plant height, fertility and yield. It will therefore be necessary to restore the 2DS chromosome arm in rec. #74 before the resistance can be used commercially. During meiosis the pairing of homologues is initiated from the telomeres (Schwarzacher, 1997). If the telomere of the rec. #74 chromosome 2DS had been lost, it may result in failure of chromosome pairing during meiosis. It may then not be possible to rely on recombination of chromosome arm 2DS of rec. #74 and a normal chromosome arm to replace the

deleted chromatin. The following strategy is therefore suggested to replace the deleted chromosome 2DS region in rec. #74:

(a) Select homozygotes for recombinants #74 and #37, making use of one of the diagnostic, recessive (null condition) microsatellite markers such as Barc228.
(b) Cross the two homozygous stocks and testcross the $F_1$ (Figure 3.22) with CSN2DT2A or CSN2DT2B.
(c) Screen the testcross $F_1$ plants with any of the microsatellite markers Gwm484, Gwm261 or Barc124 (Gwm484 scores the easiest on 2% agarose) and select plants that amplify the wheat allele.
(d) Screen these plants with *Xsopw7* that maps just outside the translocation breakpoint on 2DL of translocation rec. #74. Select plants that amplify the wheat allele (absence of diagnostic 700 bp band).
(e) Confirm the resistance.

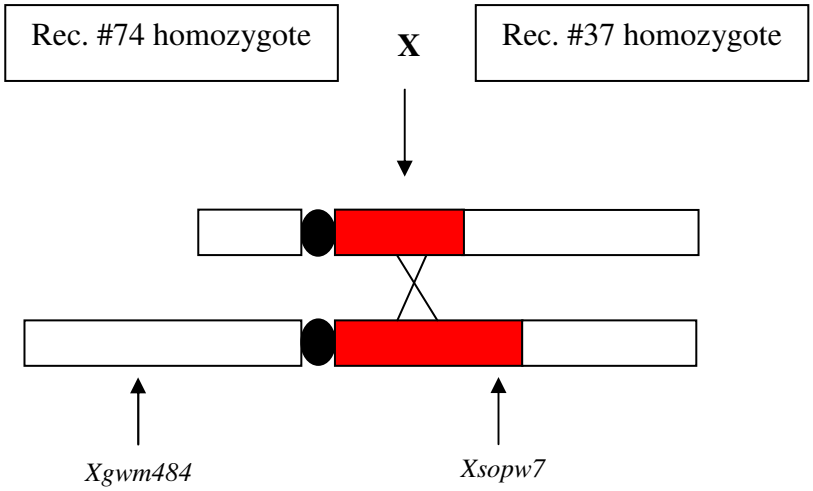



Figure 3.22 The strategy suggested to replace the deleted chromosome 2DS region in rec. #74.

### 3.5 Testing for the presence of a photoperiod insensitivity gene (*Ppd*) on the S14 translocation

Marais *et al*. (2005) transferred genes for leaf rust and stripe rust resistance from *Ae. kotschyi* to a CS background thus producing the S14 translocation. Plants with the translocation were significantly shorter and flowered earlier than the parents which raised the possibility that height reducing and photoperiod insensitivity genes could be associated with the translocation. Five genotypes, including the two parental lines, CS

and CS-S, line W84-17, as well as resistant (with the translocation) and susceptible (without the translocation) plants identified within the segregating CS-S14 translocation line, were therefore subjected to a short day (8 hours light, 16 hours dark) and long day (14 hours light, 10 hours dark) treatment in an attempt to determine if a gene for photoperiod insensitivity is in fact associated with the translocation. CS-S14 plants were tested for the presence of *Lr54* (resistant to UVPrt8) and divided into a resistant group, with the translocation, and a susceptible group, without the translocation (control). The results obtained with the experiment are summarized in Table 3.9 and Fig. 3.23 and show the average number of days to flowering for each genotype and the genotypic response to the variation in photoperiod.

Table 3.9 The average duration of time to flowering for five genotypes when subjected to short day (8 hours light, 16 hours dark) and long day (14 hours light, 10 hours dark) treatments.

| **Line** | **Number of plants per treatment** | **Average days to flowering** | | **Delay in flowering (days)** |
|---|---|---|---|---|
| | | **Long day** | **Short day** | |
| CS-S14 (-*Lr54*) | 4 | 35.25 | 48 | 12.75 |
| CS-S14 (+*Lr54*) | 7 | 37.29 | 56.86 | 19.75 |
| CS | 8 | 83.75 | 156 | 72.25 |
| CS-S | 8 | 50.25 | 111.25 | 61 |
| W84-17 | 8 | 43.38 | 82.88 | 39.5 |

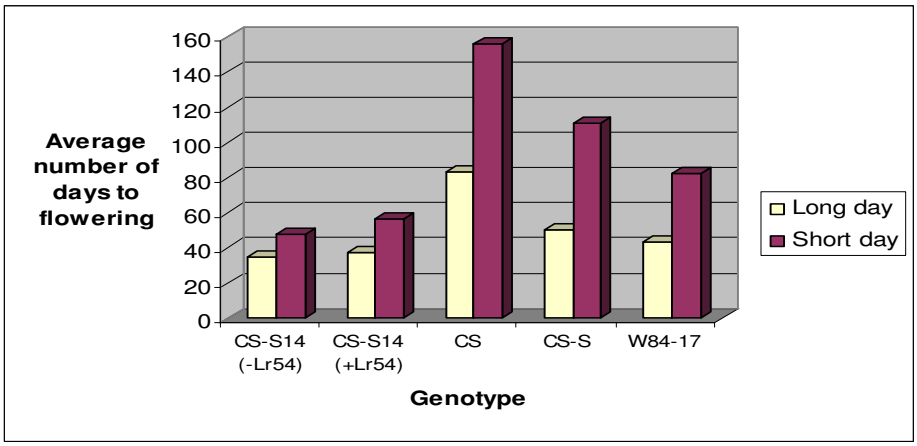



Figure 3.23 Differences in the average number of days to flowering of five genotypes subjected to long day and short day treatments at a continuous temperature of 20 degrees centigrade.

The two data sets (long day treatment and short day treatment) were analyzed separately. Visual inspection of the total data suggested that the entry means and variances might be correlated. When the correlation between the entry means and standard deviation of a mean was calculated, it was not significant ($r = 0.61$, $df = 9$, $\alpha = 0.05$); yet in view of the high correlation coefficient and the small number of degrees of freedom it was decided to do separate ANOVAs (analyses of variance) (Table 3.10 A; B).

Table 3.10 Results obtained following ANOVA of (A) the short day treatment data and (B) the long day treatment data of the photoperiod experiment.

**A**

| **Source** | **DF** | **Mean Square** | **F Value** | **Pr > F** |
|---|---|---|---|---|
| **Genotypes** | 4 | 12988.07768 | 168.59 | < .0001 |
| **Error** | 30 | 77.04107 | | |

**B**

| **Source** | **DF** | **Mean Square** | **F Value** | **Pr > F** |
|---|---|---|---|---|
| **Genotypes** | 4 | 2752.12232 | 345.38 | < .0001 |
| **Error** | 30 | 7.96845 | | |

ANOVA of the short and long day treatments showed significant differences among entry means for both photoperiods. Comparisons of entry mean values showed that significant differences ($\alpha = 0.05$) existed between the genotypes of each treatment with the exception of the difference between CS-S14 (resistant) and CS-S14 (susceptible) which was non-significant. This applied to both the long day and the short day experiments. This means that the CS-S14 plants (with and without the translocation) were always earlier flowering than CS, CS-S and W84-17 as was observed by Marais *et al*. (2005). However, this difference clearly cannot be associated with the translocation and must be the result of recombination among earliness genes on chromosome regions other than 2DL, and contributed by the common wheat parents CS and CS-S (CS-S is a near-isogenic derivative of CS which has *Rht-B1b* derived from Inia 66).

The genotypes CS and CS-S showed delays of 72.25 and 61 days in flowering, respectively, when exposed to a shorter photoperiod. W84-17 proved to be less affected when exposed to a shorter photoperiod and an average delay of 39.5 days was observed when it was grown under shorter days. However, both the resistant and susceptible S14 lines showed the smallest delay in flowering with the susceptible line being the least sensitive to a reduction in photoperiod. T-test comparisons were done of the long and short day means of each of the five entries. The results showed significant differences ($\alpha = 0.05$) between the long day mean and the short day mean of each of the five entries.

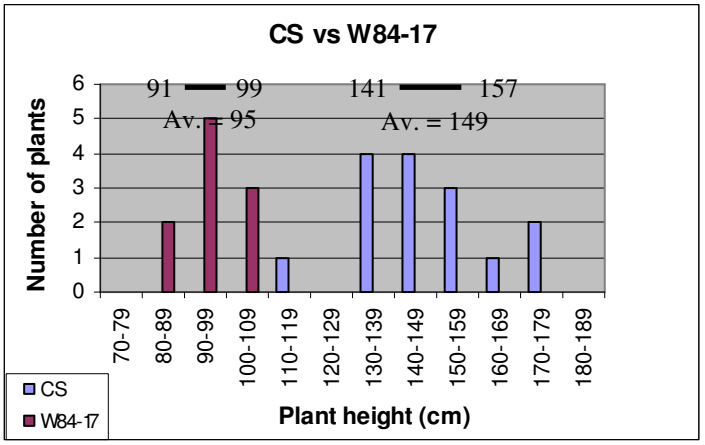
To conclude, significant differences in days to flowering were caused by the two photoperiod treatments. However, the non-significant comparisons between CS-S14 (susceptible) and CS-S14 (resistant) in both treatments, as well as the similar size (even slightly smaller in CS-S14 lacking the translocation) of the response to increased day lengths in the two genotypes did not provide proof of a gene for photoperiod insensitivity on the translocation. The earlier flowering habit of CS-S14 plants compared to CS and CS-S is therefore probably due to the effect of recombined background genes.

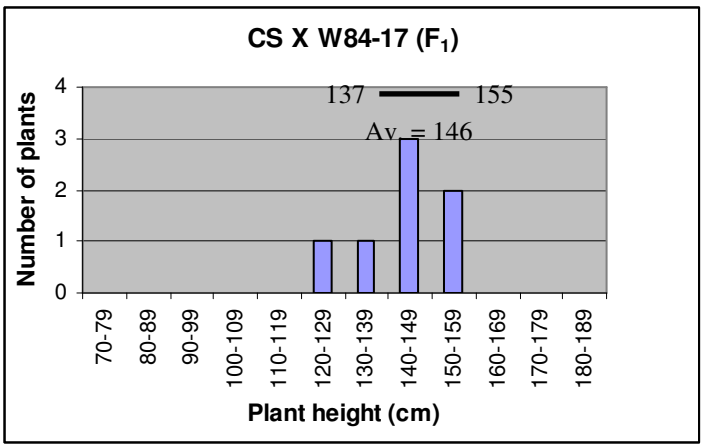
### 3.6 Confirmation of a height reducing gene (*Rht*) associated with the translocation

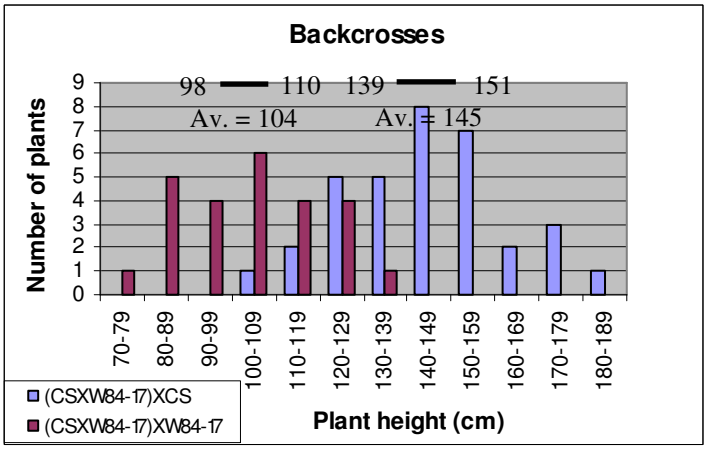
When Marais *et al*. (2005) produced the S14 translocation they suggested that the translocation may be associated with a dominant height reducing gene (*H*) due to the short stature of some of the plants containing the S14 translocation. In an attempt to confirm this, a series of crosses and backcrosses were conducted which involved a control group (crosses of CS and W84-17) (Fig. 2.3a) and an experimental group (crosses of CS and $F_1$:0514 – a W84-17 near-isogenic line having the S14 translocation) (Fig. 2.3b). The material also provided a preliminary opportunity for testing the possibility that apart from the resistance, the translocation may carry agronomically detrimental/advantageous genes. Therefore, in addition to plant height, the time to ear emergence, time to flowering, 100 kernel mass and plant yield were also determined for individual plants.

The distribution of plant height measurements in the parental lines, $F_1$ backcross and $F_2$ populations of the control and experimental groups are depicted by means of histograms in Fig. 3.24 and Fig. 3.25, respectively. The average height and (where applicable) a 95% confidence interval were also calculated for each population. The expected genotypes and their frequencies for each population have previously been provided in Fig. 2.3a and Fig. 2.3b, respectively, and should be considered in conjunction with the observed height measurements that are given here. The histogram of the parental lines, CS and W84-17 (Fig. 3.24), showed that they are clearly divided into separate groups with average heights of 95 cm (*Rht-B1b Rht-B1b hh*) and 149 cm (*Rht-B1a RhtB1a hh*), respectively. The $F_1$ (*Rht-B1a Rht-B1b hh*) distribution was similar to that of CS confirming that the *Rht-B1a* allele is dominant. The height distributions of the $F_2$ and the two $BF_1$ populations are in accord with those of the parents and $F_1$. With the exception of one backcross plant, the plants in all the groups were taller than 80 cm. Only one backcross plant was taller than 180 cm (185 cm).

When the plant heights of the parental lines in the experimental group (Fig. 3.25) were analyzed, two separate groups with average plant heights of 71 cm ($F_1$:0514 – *Rht-B1b Rht-B1b Hh*) and 149 cm (CS – *Rht-B1a Rht-B1a hh*) were respectively observed. When compared to the control group, the plants in the shorter class were, on average, 24 cm shorter than W84-17. This would suggest that the height reducing gene, *H,* shortens the plant stature, even in the heterozygous state. The 95% confidence interval of $F_1$:0514 was larger than that of CS (as well as that of W84-17 in the control group) primarily as a result of a single outlier (27 cm). However, even when omitting this plant, the near isogenic $F_1$ plants are clearly shorter than W84-17. Comparison of average $F_1$ plant heights in the control and experimental groups showed that the experimental group was, on average, eight centimeters shorter. When the $F_1$ of the experimental group was backcrossed to CS and $F_1$:0514, a much wider distribution of plant heights was observed for both backcrosses and the $F_2$ population. However, as in the control group, plants were never taller than 180 cm; on the other hand many were shorter than 80 cm. When comparing the results obtained (Figures 3.24 and 3.25) with the expected genotypes, assuming that the resistance is linked to a dominant gene for reduced height (Figures 2.3a and 2.3b), it is evident that such a gene does in fact occur on the translocation.



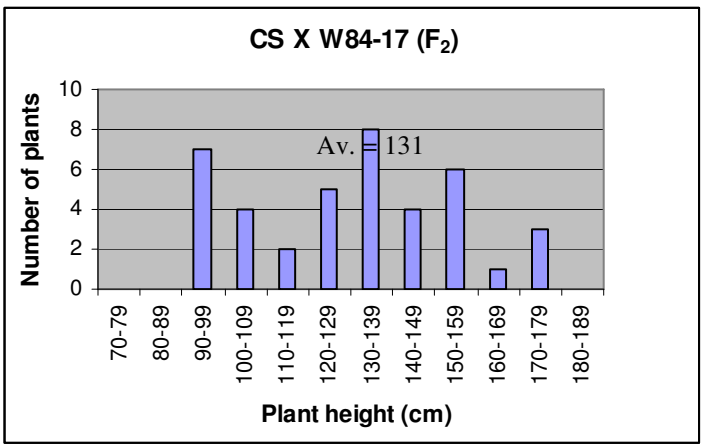




Figure 3.24 Plant heights of the parents, $F_1$, $F_2$ and backcross progenies in the control group (Fig. 2.3a). Where applicable the 95% confidence intervals are given above the histogram.

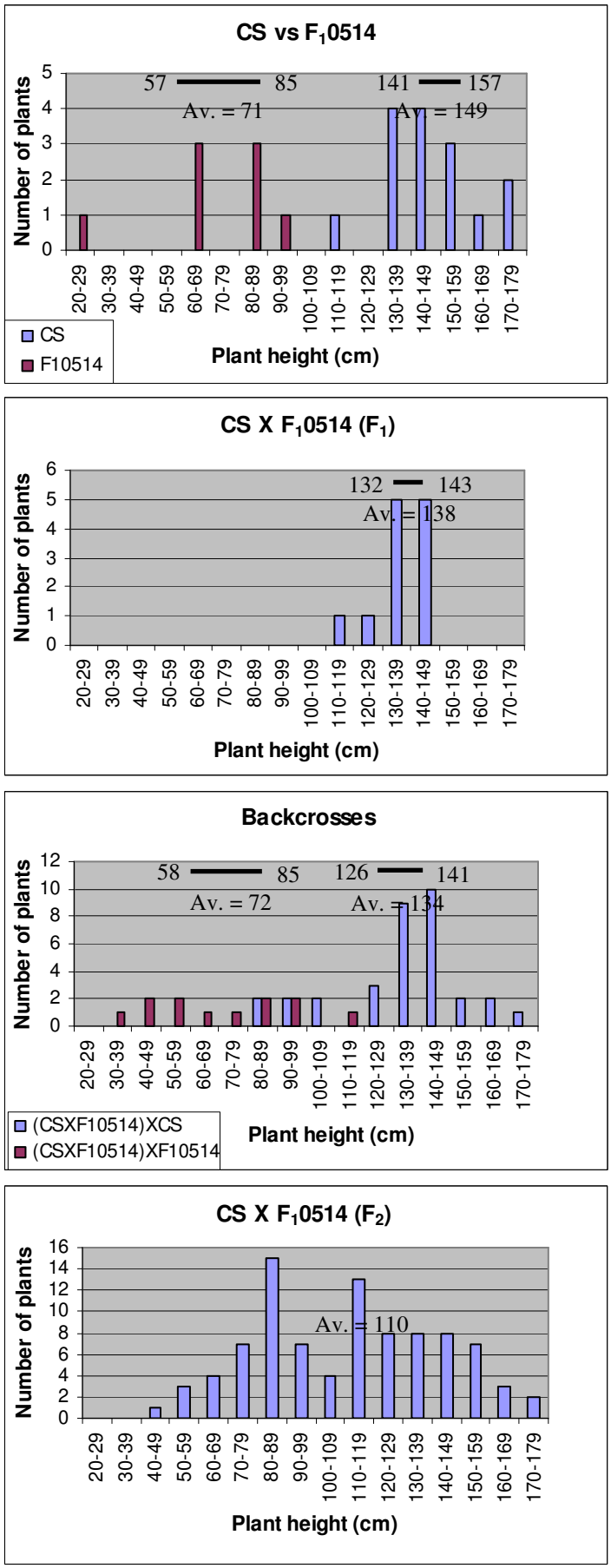



Figure 3.25 Plant heights of the parents, $F_1$, $F_2$ and backcross progenies in the experimental group (Fig. 2.3b). Where applicable the 95% confidence intervals are given above the histogram.

T-test comparisons were also done between the $F_2$ progeny of the control and experimental groups and between the resistant and susceptible plants within the experimental group. The results showed a significant difference between the $F_2$ control (average = 130.7 cm) and $F_2$ experimental groups (average = 110.3 cm) ($P<0.01$) as well as between the resistant (average = 106.6 cm) and susceptible plants (average 128.9 cm) within the $F_2$ experimental group ($P<0.05$); thus confirming the presence of a height reducing gene.

### 3.6.1 Agronomical advantages/disadvantages associated with the S14 translocation

Previous studies have shown that translocations from wild species may sometimes be associated with detrimental/beneficial genes. The species-derived chromatin does not normally pair and recombine with homoeologous wheat chromatin during meiosis, resulting in co-inheritance of the resistance and associated genes. One such example is the 1B/1R translocation that carries genes for leaf rust (*Lr26*), stripe rust (*Yr9*), stem rust (*Sr31*) and powdery mildew (*Pm8*) resistance. This translocation was found to increase the above-ground biomass, 1000-grain weight, test weight and the number of spikes per $m^2$ (Villareal *et al*., 1991), however, wheat lines carrying *Sr31* exhibit dough stickiness and have considerably shorter dough un-mixing times compared to non-*Sr31* wheats (Barnes, 1990). Another example is the *Lr19* translocation on 7D which is associated with a stem rust resistance gene, *Sr25*, as well as increased yield (13%), biomass (10%) and grains per spike (15%) when evaluated in high yielding wheat backgrounds (Reynolds *et al*., 2001). However, the *Lr19* translocation also carried an undesirable gene for yellow flour pigmentation which has been removed through allosyndetic pairing induction but which unfortunately also resulted in the loss of *Sr25* (Marais, 1992). Studies by Butler *et al*. (2002), Singh *et al*. (2001) and Nizam Uddin and Marshall (1989) have shown that dwarfing genes may result in higher or lower yields depending on environmental conditions.

The data obtained in the $F_2$ experimental group were used to calculate correlations among all the characteristics that were measured on single plants. The correlations were calculated separately for the resistant and susceptible $F_2$ plants and the results obtained are listed in Table 3.11.

Table 3.11 Correlation coefficients between plant characteristics for plants with (resistant - blue; 71 plants) and without the translocation (susceptible - red; 15 plants) in the $F_2$ plant height experimental population.

| | **Plant height (cm)** | **Days to ear emergence** | **Days to flowering** | **100 kernel mass (g)** | **Yield (g)** |
|---|---|---|---|---|---|
| **Plant height (cm)** | 1 | 0.02 | -0.02 | 0.54 (*) | 0.78 (*) |
| **Days to ear emergence** | 0.14 | 1 | 0.99 (*) | -0.005 | -0.06 |
| **Days to flowering** | 0.07 | 0.99 (*) | 1 | -0.04 | -0.09 |
| **100 kernel mass (g)** | 0.45 | -0.15 | -0.15 | 1 | 0.43 (*) |
| **Yield (g)** | 0.77(*) | 0.38 | 0.31 | 0.29 | 1 |

*Significant at 0.05 level

A significant positive correlation was found between plant height and plant yield and between plant height and 100 kernel mass in the resistant group. For the susceptible group plant height showed significant correlation with plant yield while the correlation of plant height and 100 kernel mass was relatively high but not significant. These results are not unexpected as the plants were grown under non-limiting greenhouse conditions. Taller plants accumulated more photosynthate that could be translocated to the seeds. Thus, the associated height reducing gene is detrimental to yield under greenhouse conditions; yet this will not necessarily be the case under all commercial growing conditions.

In an attempt to reduce the overriding effect of plant height when trying to evaluate the S14 translocation for possible associated effects, the results obtained with resistant and susceptible plants were organized into three plant height categories. The average for days to ear emergence, days to flowering, 100 kernel mass and plant yield were then calculated for each category (Table 3.12).

Table 3.12 The averages of plant characteristics for three plant height categories of resistant (A) and susceptible (B) plants within the $F_2$ plant height experimental population.

**A**

| Plant height (cm) | Number of plants | Average plant height (cm) | Days to ear | Days to flower | 100 kernel mass (g) | Yield (g) |
|---|---|---|---|---|---|---|
| 40-79 | - | - | - | - | - | - |
| 80-119 | 5 | 97.6 | 84.2 | 93.8 | 4.45 | 17.06 |
| 120-179 | 10 | 144.5 | 86.8 | 96.1 | 5.01 | 34.34 |

**B**

| Plant height (cm) | Number of plants | Average plant height (cm) | Days to ear | Days to flower | 100 kernel mass (g) | Yield (g) |
|---|---|---|---|---|---|---|
| 40-79 | 12 | 68.17 | 90.92 | 102.25 | 4.60 | 0.92 |
| 80-119 | 33 | 96.52 | 90.03 | 100.21 | 5.08 | 8.04 |
| 120-179 | 26 | 142.54 | 90.27 | 100.04 | 5.54 | 24.41 |

Since susceptible plants do not contain the translocation and height reducing gene, no plants were shorter than 80 cm. Average days to ear emergence and days to flowering did not differ significantly ($P>0.05$) for the corresponding height categories of resistant and susceptible plants. This supports the earlier conclusion that the translocation is not associated with a gene for photoperiod insensitivity. Both the resistant and susceptible groups showed a slight increase in 100 kernel mass as plant height increases. Resistant plants in the 80-119 cm (non-significant) and 120-179 categories (significant, $P<0.05$) had a higher 100 kernel mass compared to susceptible plants in the corresponding categories which suggests that the translocation increased the 100 kernel mass. When the yields of plants in the different height categories were compared for resistant and susceptible groups, the susceptible plants had significantly higher yields. This would suggest that in addition to the effect ascribable to the height reducing gene, the translocation may have a further detrimental effect on yield. However, since the sample size of the $F_2$ experimental susceptible plants was very small, these conclusions must be regarded as preliminary.

### 3.7 Mapping of a height reducing gene (*H*) on the translocation

Following the confirmation of a height reducing gene on the translocation, an attempt was made to map *H*. In order to be able to characterize the group of 10 recombined translocations for the presence/absence of *H*, it was necessary to assign height genotypes to the material derived in the allosyndetic pairing induction experiment (Fig. 3.26).

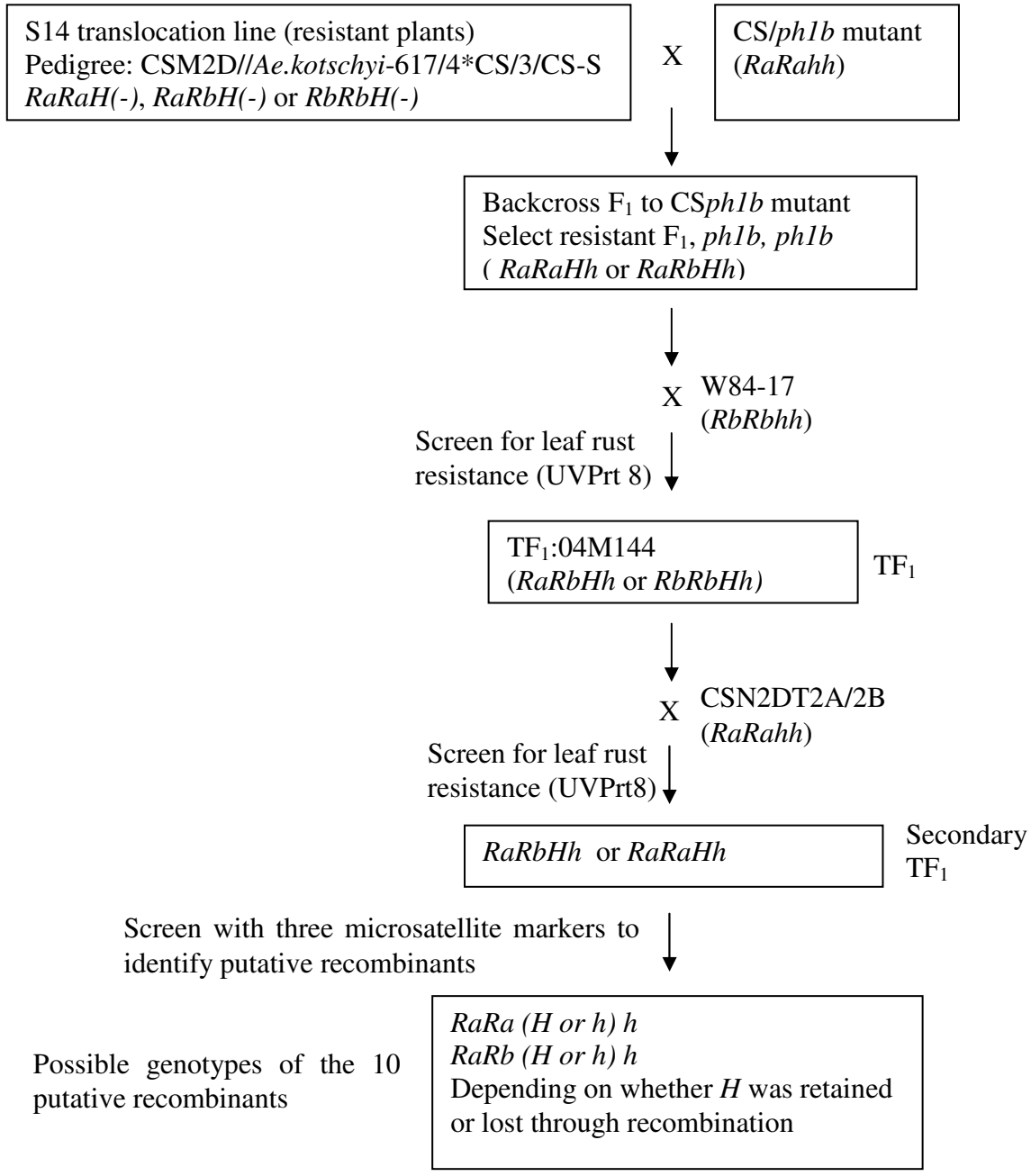



Figure 3.26 Height genotypes of the material used in the allosyndetic pairing induction experiment and possible genotypes of the 10 recombinants. The meanings of the symbols are as follows: *Ra* = *Rht-B1a*; *Rb* = *Rht-B1b*; *H* = height reducing gene that occurs on the S14 translocation; *h* = corresponding height reducing allele in wheat.

If *H* was retained on the translocation, and it derived from a plant that carried *Rht-B1b* (frequency = 9/16), the secondary $TF_2$ progeny would include dwarfs. However, if homoeologous recombination removed *H* from the translocation, then the particular $TF_2$ progeny would not contain dwarfs (Fig. 2.4). The adult plant heights of resistant secondary $TF_1$, resistant secondary $TF_2$ and resistant/susceptible secondary $TF_3$ recombinant lines and control lines CS (*Rht-B1a Rht-B1a hh*), W84-17 (*Rht-B1b Rht-B1b hh*) and CS-S14 (*Rht-B1a Rht-B1a H(-), Rht-B1a Rht-B1b H(-)* or *Rht-B1b Rht-B1b H(-))* were measured and are listed in Table 3.13. The secondary $TF_1$ and $TF_2$ plants were grown in a greenhouse whereas the secondary $TF_3$ plants were grown under dry land conditions in the field.

The data suggested that *H* is still present in recombinants 119, 205, 247 and 256(?). In the remaining recombinants *H* may either have been replaced by the recessive common wheat allele, *h*, or the presence of *H* was masked by the presence of *Rht-B1a*. In an attempt to confirm whether the shortest recombinant retained *H*, resistant (20 plants) and susceptible (11 plants) $BCF_3$ progeny segregating for rec. #74 and the control lines CS, W84-17, CS-S and CS-S14 (resistant and susceptible plants) were evaluated for adult plant height (Table 3.14). The heights of the plants recorded in Table 3.14 were considerably shorter than the comparable heights measured in previous experiments (Table 3.13). This was due to the fact that the plants were grown under supplemented light (total day light hours = 16h) in an un-cooled greenhouse. The phenotypic data of CS, CS-S and W84-17 appeared to correlate well with their respective *Rht-B1* genotypes. Plants in the CS-S14 resistant and susceptible groups probably were *Rht-B1b Rht-B1b* homozygotes. However, they were still considerable shorter than the two *Rht-B1b Rht-B1b* controls (W84-17 and CS-S). The CS-S14 resistant lines were on average 7 cm shorter than the CS-S14 susceptible group which probably related to the presence of *H* in the resistant segregates that carried the full translocation. Background genes affecting height and derived from the parental wheat lines must be responsible for the height difference between CS-S14 and the control lines W84-17 and CS-S. A comparable difference in days to flowering of the same groupings was seen in the photoperiod response experiment (Table 3.9; Figure 3.23). The earlier flowering habit of the CS-S14 derivatives could have contributed to their comparatively shorter stature.

Table 3.13 Plant heights of resistant $TF_1$, $TF_2$ and $TF_3$ plants derived from each of 10 recombinant lines, together with the heights of control lines.

| Line | $TF_1$ plant height (cm) (Resistant – Greenhouse) | $TF_2$ plant height (cm) (Resistant – Greenhouse) | $TF_3$ plant heights (cm) (Field Grown) (Segregating or pure breeding resistance) | Inferred height genotype |
|---|---|---|---|---|
| 25 | 133 | 139 | 108, 108, 108, 109, 109, 112, 115, 118, 118, 120, 121, 122, 123, 125, 125, 126, 126, 127, 127, 127 | *hh* |
| 37 | 91, 123, 148 | 137 | 78, 86, 86, 91, 92, 94, 95, 95, 95, 96, 97, 97, 98, 99, 99, 101, 101, 104, 108, 108, 108, 112 | *hh* |
| 74 | 101, 103 | 122 | 106, 114, 114, 115, 116, 116, 116, 118, 118, 118, 119, 121, 123, 123, 126, 126, 128, 128, 129, 133 | *hh* |
| 119 | 110 | 92 | 58, 60, 62, 67, 68, 68, 69, 69, 70, 72, 74, 75, 84 | *H(-)* |
| 148 | 95, 105, 110 | 104 | 82, 84, 86, 87, 95, 96, 96, 97, 97, 98, 98, 99, 102, 102, 110, 110, 112, 113, 119, 120, 122 | *hh* |
| 205 | 96, 104, 119 | 100 | 47, 49, 63, 63, 66, 66, 67, 69, 69, 69, 70, 70, 71, 72, 72, 72, 73, 74, 75, 77, 79 | *H(-)* |
| 247 | 90, 97, 106 | 58, 98 | 51, 55, 56, 67, 71, 77, 78 | *H(-)* |
| 256 | 106, 108 | 114 | 65, 78, 81, 84, 84, 85, 85, 86, 88, 91,92, 92, 93, 95, 97, 97, 101, 101, 103, 108 | *H(-)*? |
| 265 | 89 | 98, 135 | 72, 90, 91, 92, 94, 95, 96, 97, 98, 98, 100, 100, 100, 101, 101, 102, 103, 103, 108, 111, 115 | *hh* |
| 273 | 95 | 122 | 83, 84, 90, 92, 98, 100, 103, 107, 110, 113, 113, 115, 116 | *hh* |
| CS (*RaRahh*)[1] | 133, 137, 144, 145, 148, 150, 165 | | 86, 101, 107, 110, 112, 115, 116, 116, 118, 119, 119, 120, 120, 122, 123, 125, 126, 128 | |
| W84-17 (*RbRbhh*)[1] | 84, 89, 94, 96 | | 73, 73, 75, 76, 78, 78, 78, 78, 79, 81, 83, 83, 83, 85, 85, 86, 93 | |
| CS-S14 (*RaRaH(-)*; *RaRbH(-)*; *RbRbH(-)*)[1] | 41, 45, 48 | | 53, 53, 56, 61, 64, 65, 70, 70, 78, 80, 80, 85, 86, 87 | |

[1] The meanings of genotypic symbols are as follows: *Ra* = *Rht-B1a*; *Rb* = *Rht-B1b*; *H* = Height reducing gene that occurs on the S14 translocation; *h* = wheat 2DL chromatin region corresponding to the *H* locus.

 <70 cm  70 – 99 cm  > 99 cm

Table 3.14 Plant height phenotypic data for $BCF_3$ (cross = CS-S14 translocation/2*CS*ph1b* mutant /W84-17/3/CSN2DT2A-B/4/2*W84-17) progeny segregating for recombinant #74.

| **Plant nr.** | ***Rht-B1* genotype** | **Plant height (cm)** | **Average plant height (cm)** |
|---|---|---|---|
| 1 R | | 73 | |
| 2 R | | 73 | |
| 3 R | | 65 | |
| 4 R | | 71 | |
| 5 R | | 71 | |
| 6 R | | 74 | |
| 7 R | | 60 | |
| 8 R | | 68 | |
| 9 R | | 57 | |
| 10 R | | 61 | |
| 11 R | | 74 | |
| 12 R | | 91 | |
| 13 R | | 61 | |
| 14 R | | 53 | |
| 15 R | | 56 | |
| 16 R | | 56 | |
| 17 R | | 80 | |
| 18 R | | 77 | |
| 19 R | | 65 | |
| 20 R | | 72 | |
| **Average for R plants** | | | **67.9** |
| 1 S | | 76 | |
| 2 S | | 87 | |
| 3 S | | 80 | |
| 4 S | | 79 | |
| 5 S | | 51 | |
| 6 S | | 55 | |
| 7 S | | 66 | |
| 8 S | | 64 | |
| 9 S | | 56 | |
| 10 S | | 75 | |
| 11 S | | 80 | |
| **Average for S plants** | | | **69.9** |
| CS | *RaRa* | 97, 97, 98, 99, 102, 102, 107, 107, 109, 110 | **102.8** |
| CS-S | *RaRb* | 73, 75, 75, 77, 79, 83, 83, 84, 90, 92 | **81.1** |
| W84-17 | *RbRb* | 57, 70, 75, 76, 77, 78, 78, 79, 79, 82 | **75.1** |
| CS-S14 (R) | *RbRb, RaRb* | 31, 39, 40, 40, 40, 41, 42, 43, 44, 44 | **40.4** |
| CS-S14 (S) | *RbRb, RaRb* | 41, 44, 46, 59 | **47.5** |

When the average plant heights of plants segregating for the presence of rec. #74 were compared it seemed that, on average, the resistant group was 2 cm shorter than the susceptible group. No plants in the resistant and susceptible groups exceeded 99 cm. The shortest plant in the resistant group was 53 cm tall and in the susceptible group the shortest plant was 51 cm. The very similar height distribution in both groups suggested that *H* is no longer associated with rec. #74.

### 3.8 Mapping of *Lr54* and *Yr37* on the translocation

Hemizygous leaf rust resistant $TF_1$ progeny were screened with microsatellite markers to select 10 recombinants of which rec. #74 was the shortest. $TF_2$ progeny of the 10 recombinants were screened by Prof. Z.A. Pretorius (University of the Free State, South Africa) for stripe rust resistance using *P. striiformis* pathotype 6E22A+. The recombinants were screened twice with 4-8 plants included in each replicate. The number of plants employed and the infection types observed are summarized in Table 3.15. Also shown is the response of control line CS-S14 (containing *Lr54* and *Yr37*) and the susceptible lines CS, CS-S and W84-17 (Fig. 3.27).

The individual plants among the progeny of a recombinant would have included nullisomics, monosomics and disomics for chromosome 2D (recombined). The individual plants will also have included disomics, trisomics and tetrasomics for either of chromosomes 2A or 2B. As a result the infection types varied from the typically strong infection type that occurs in the segregating S14 translocation line to the very susceptible infection type produced in the controls. All the recombinants included plants that were stripe rust resistant, however, only recombinants 25, 74, 119, 148, 205 and 256 produced the very strong hypersensitive reaction characteristic of *Yr37*. Recombinants 37, 247, 265 and 273 produced infection type 2 which is most probably also due to the presence of *Yr37*, yet the slightly milder expression may have been caused by the associated aneuploidy. Recombinant #74 retained both the leaf rust (*Lr54*) and stripe rust (*Yr37*) resistance genes. Being the shortest recombinant with only the most proximal region consisting of foreign chromatin it suggests that *Lr54* and *Yr37* are located towards the centromere.

Table 3.15 Results obtained following screening of control lines and translocation recombinants with *P. striiformis* pathotype 6E22A+. Plants with an infection type lower than 3 were considered resistant. The number of plants is indicated first followed by the particular infection type (data provided by Prof. ZA Pretorius, Dept. of Plant Sciences, University of the Free State).

| **Lines** | **Number of plants and infection type Replicate 1** | **Number of plants and infection type Replicate 2** |
|---|---|---|
| CS | Infection type 4 | Infection type 4 |
| CS-S | Infection type 4 | Infection type 4 |
| W84-17 | Infection type 4 | Infection type 4 |
| CS-S14 | 1p;c/2p;1cn/2p4 | 1p;c/5p;1cn/1p4 |
| Rec. 25 | 1p;1c | 1p;1c/1p3+ |
| Rec. 37 | 3++c/4 | 2p2c/4p3++ |
| Rec. 74 | 2p2+c/5p3++ | 1p;1/1p3c/4p4 |
| Rec. 119 | 1p3c/5p4 | 2p;c/3p3++ |
| Rec. 148 | 3p3c/2p3+ | 3p;1c/1p;1+c/1p3 |
| Rec. 205 | 5p;1+c | 2p;1+c/4p;1c |
| Rec. 247 | 2p2 | 1p;/1p2/2p3- |
| Rec. 256 | No plants | 1p;cn/3p3 |
| Rec. 265 | 2p2/4p3+ | 2p2 |
| Rec. 273 | 1p2/1p4 | 3p2/1p3c |

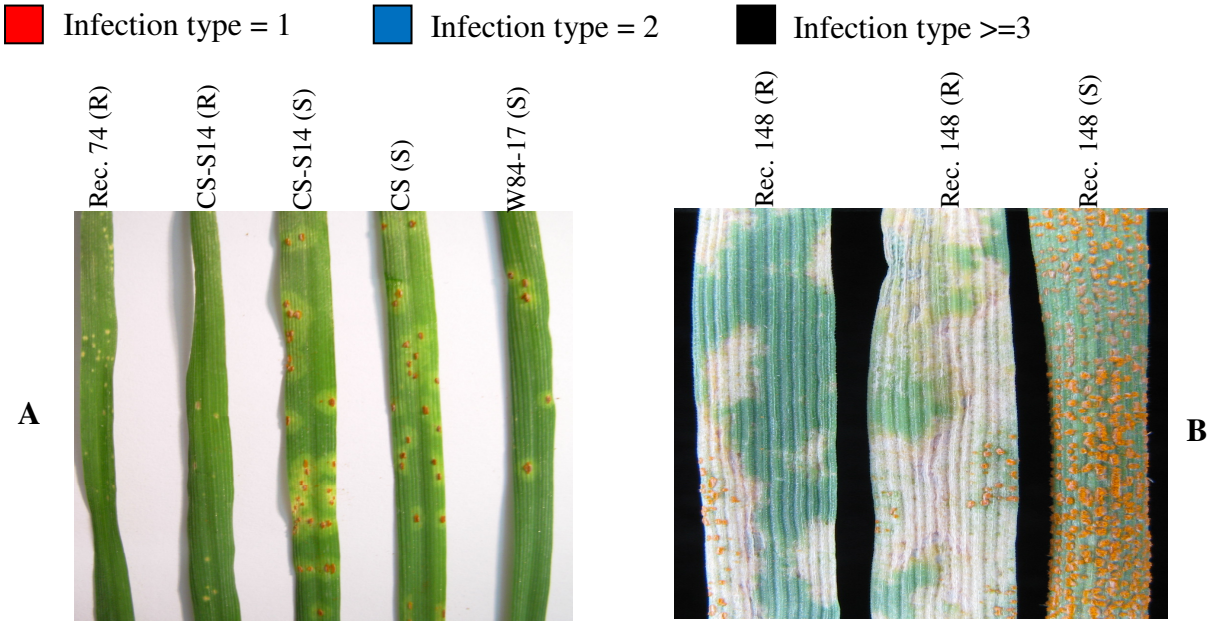



Figure 3.27 (A) Seedling response of $F_2$ plants with *Lr54* (R) and plants lacking the gene (S) following infection with *P. triticina* pathotype UVPrt8. (B) Seedling response of $F_2$ progeny with (R) and without (S) *Yr37* following infection with *P. striiformis* pathotype 6E22A+.

### 3.9 Concluding remarks

The primary objective of this study was to search for recombinants of the *Lr54*/*Yr37* translocation and then to characterize, map and compare these with reference to the original, full-sized translocation. It was of particular interest to determine whether height reducing and photoperiod response genes do in fact occur on the full-length translocation and whether it would be possible to recover resistant recombinants that lack these. A final goal was to develop a dominant PCR marker that can be used in marker assisted selection of the *Lr54*/*Yr37* resistance genes in breeding programs.

Ten putative translocation recombinants were identified following the screening (with three microsatellite markers) of 159 resistant 04M144 testcross plants, each hemizygous for the translocation. The 10 recombinants were further characterized with five more microsatellite markers and the data were used to group them in two different size categories of which rec. #74 was the shortest. The data obtained for marker *Xcfd50* showed that this locus was duplicated in two recombinants whereas the position of marker *Xgdm6* appeared to be inconsistent with its mapped location (Sourdille *et al*., 2004). It furthermore seemed highly likely that *Xgdm6* had been deleted in six of the 10 recombinants. The data also appeared to be inconsistent with the very low level of multiple crossovers that could be expected to occur between alien and wheat chromosome segments in homoeologous pairing induction experiments. Analysis of the results suggested a plausible explanation. Two successive crosses were used to derive the recombinants and these involved the wheat parents CS*ph1b* and W84-17, respectively. It appears that the CS*ph1b* and W84-17 chromosome arms 2DL differ with respect to the order of the marker loci *Xgdm6* and *Xcfd50*. This difference was probably brought about by intra-chromosomal translocation of either locus. Meiotic pairing between the homoeologous translocation recombinants (that each exchanged a distal section of the *Ae. kotschyi* translocation for CS*ph1b* 2DL chromatin) and 2DL of W84-17 resulted in irregularity of the bivalents (unpaired compensation loops were formed) in areas affected by the translocation. Single crossovers within either of two alternative pairing structures could explain the origin of each recombinant. While the aberrations resulted in the formation of *Xcfd50* duplication and *Xgdm6* deletion gametes, these structural differences will not affect the utility of the recombinants as the affected region is

located well beyond the *Ae. kotschyi* – wheat translocation break point. During backcrossing the *Xgdm6*/*Xcfd50* locus order of the recurrent parent will be established.

In an attempt to find further markers with which to genetically map the recombinants and to derive a dominant PCR marker linked to the shortest translocation recombinant (rec. #74); RAPD, AFLP, RGA and SCAR polymorphisms were evaluated. Twelve RAPD primers and nine degenerate RGA primers produced no useful polymorphic loci. Two previously described SCAR loci, *XustII-2J*$_1^d$ and *Xsopw7*, mapped to the translocation but did not occur in rec. #74. In an AFLP-based search for useful polymorphisms, three labelled *Eco*RI primers (ACA, AGG, AAC) were used in combination with five unlabeled *Mse*I primers (CAG, CTG, CAT, CTC, CTA). Four selective *Mse*I primers amplified six polymorphic fragments associated with the S14 translocation of which only one (*Eco*RI – AAC/*Mse*I – CAT 410) was specific for the shortest translocation recombinant and was converted into a SCAR marker (SCAR-410). SCAR-410 was confirmed on resistant and susceptible $BCF_2$ and $BCF_3$ progeny segregating for rec. #74 as well as on the panel of 10 recombinants. Being present in the shortest recombinant, *Xscar410* also occurs in the remaining nine recombinants. This marker will therefore be very effective in marker assisted selection for the identification of lines carrying *Lr54*. In addition to the markers used to identify and characterize the translocation, four microsatellite markers specific for chromosome arm 2DS were used to confirm that the translocation is restricted to chromosome arm 2DL as was suggested by Marais *et al*. (2005). Three of the markers mapped to the most proximal deletion bin on chromosome 2DS whereas the remaining marker mapped to the most distal deletion bin that included the 2DS telomere (Sourdille *et al*., 2004). Each of the four markers amplified the wheat allele in a control line hemizygous for the full-lenght translocation, which confirmed that the translocation was probably confined to chromosome arm 2DL. When the 10 translocation recombinants were screened with the 2DS markers, the data allowed for mapping of the four loci relative to one another which suggested that *Xbarc124* occurs between *Xgwn484* and *Xcfd116* rather than distally from them as suggested by the literature. The order of the four loci were: telomere – *Xgwm261* – *Xbarc124* – *Xgwm484* – *Xcfd116* – centromere. Three markers amplified a null allele in rec. #74 which indicates a large deletion. The deletion could involve either a major intercalary

region or the distal end of chromosome arm 2DS. Depending on the size of the deletion it may affect plant phenotype characteristics and it will therefore be necessary to restore chromosome arm 2DS of the translocation. The most logical way to achieve this would be through ordinary homologous pairing and crossing over between the alien regions of rec. #74 and one of the longer translocation recombinants, such as #37. Towards this end, homozygotes of recombinants #74 and #37 can be crossed and the heterozygous $F_1$ testcrossed with CSN2DT2A/2B. The $TF_1$ progeny can then be screened with chromosome arm 2DS specific markers and *Xsopw7* that maps just outside the translocation breakpoint on 2DL of rec. #74 in order to recover the desired recombinant.

Marais *et al*. (2005) found that S14 segregates were shorter and earlier flowering than the parents and suggested that height reducing and photoperiod insensitivity genes may occur on the translocation. In order to determine whether a gene for photoperiod insensitivity is associated with the translocation, a panel of plants was exposed to a short and a long day treatment in a controlled environment chamber. However the data showed that the photoperiod response of plants with and without the translocation did not differ significantly. A series of crosses were also made to test for the presence of a height reducing gene. Control (CS X W84-17) and experimental (CS X $F_1$:0514) crosses were made and the respective $F_1$ plants were backcrossed to both parents. Plant height measurements on the parents, $F_1$, $F_2$ and $BF_1$ for both groups showed that plants in the experimental group were shorter than those in the control group. T-test comparisons of resistant and susceptible $F_2$ plants in the experimental group also showed a significant difference ($P<0.01$). It was therefore concluded that a height reducing gene (*H*) is associated with the translocation. The height reducing effect of *H* was much more pronounced in the absence of *Rht-B1a* and in *Rht-B1b* homozygotes the added presence of *H* resulted in dwarf plants. A preliminary attempt was also made to determine whether the S14 translocation may contain any advantageous/detrimental genes. The days to ear emergence, days to flowering, 100 kernel mass and total plant yield were therefore also determined for the experimental $F_2$ population. A significant positive correlation was found between plant height and plant yield and between plant height and 100 kernel mass for plants in the resistant group. However, these plants were grown under non-limiting conditions where tall plants were at an advantage. Furthermore, segregation at the

*Rht-B1* locus obviously made a major contribution towards the observed differences in plant height, plant yield and 100 kernel mass. In order to remove the confounding effect of plant height, resistant and susceptible plants were divided into three height categories and the average days to ear emergence, days to flowering, 100 kernel mass and total plant yield were derived for each category. The data suggested that the translocation increased 100 kernel mass but had a detrimental effect on total plant yield. However, these observations applied to the full length translocation. Once its 2DS arm has been reconstructed, near-isogenic bulks with, and without, the shortened translocation #74 should be evaluated more carefully under commercial growing conditions.

In an attempt to map *H*, resistant secondary $TF_1$, $TF_2$ and $TF_3$ progeny of the 10 recombinant lines were evaluated for plant height. If the progeny included dwarf plants it was taken as an indication of the presence of *H*. However, a negative result (absence of dwarfs) was inconclusive as it could also have been the result of segregation of *Rht-B1a*. Four recombinants produced dwarfs among their progeny and thus retained *H*. The progeny of the shortest recombinant did not include dwarfs. To confirm that it had lost *H*, resistant (20 plants) and susceptible (11 plants) $BCF_3$ (CS-S14 translocation/2*CS*ph1b* mutant//W84-17/3/CSN2DT2A-B/4/2*W84-17) progeny of rec. #74 were evaluated for plant height. When the average plant heights of the susceptible and resistant groups were compared, the resistant group was only 2 cm shorter. The plant height distributions within the two groups were very similar and suggested that *H* was no longer associated with rec. #74. The data suggested that *H* occurs between the #74 and #37/#205 breakpoints on the full-length translocation.

In an attempt to map *Yr37*, $TF_2$ progeny of each recombinant was screened for stripe rust resistance using *P. striiformis* pathotype 6E22A+. All the recombinants included resistant progeny. Six recombinants expressed the very low infection type associated with the S14 translocation, whereas the remainder showed a less severe response which could relate to aneuploidy of the group 2 chromosomes. Since rec. #74 retained both the rust resistance genes, *Lr54* and *Yr37*, these genes must be located near the centromere.

In conclusion, a translocation recombinant (#74) that retained *Lr54* and *Yr37* was selected. The #74 translocation is considerably shorter than the full-length translocation and is located proximally on 2DL. While it appears to carry a large 2DS deletion, the deletion occurs outside of the introgressed region. Thus, it should be possible to restore the deleted wheat chromatin through cross-breeding and selection. Molecular markers that are tightly linked to *Lr54* and *Yr37* were identified. These are suited to marker-aided selection and include a dominant, AFLP-derived SCAR marker plus three recessive (null-allele) microsatellite markers.

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# Addendum A

A summary of results obtained following the screening of resistant plants from the cross CS nullisomic 2D tetrasomic 2A/2B and 04M144 with microsatellite markers Barc228, Gdm6 and Wmc167 in an attempt to identify translocation recombinants.

| **Line** | ***Xbarc228*** | ***Xgdm6*** | ***Xwmc167*** |
|---|---|---|---|
| *Ae. kotschyi* | / | / | / |
| CS-S14 (hemi) | / | / | / |
| CS | + | + | + |
| W84-17 | +* | +* | + |
| CSN2AT2B | + | + | + |
| CSN2BT2A | + | + | + |
| CSN2DT2A/B | / | / | / |
| CSDT2DS | / | / | / |
| CSDT2DL | + | + | + |
| 1 | / | / | / |
| 2 | + | + | + |
| 3 | +* | +* | + |
| 4 | +* | +* | + |
| 5 | +* | +* | + |
| 6 | / | / | / |
| 9 | +* | + | + |
| 10 | +* | +* | + |
| 11 | +* | + | + |
| 13 | / | / | / |
| 14 | + | + | + |
| 15 | +* | +* | + |
| 16 | +* | +* | + |
| 17 | / | / | / |
| 18 | +* | +* | + |
| 19 | + | +* | + |
| 20 | +* | +* | + |
| 21 | / | / | / |
| 24 | +* | +* | + |
| 25 | / | / | + |
| 26 | + | + | + |
| 27 | / | / | / |
| 28 | +* | +* | + |
| 29 | / | / | / |
| 32 | +* | +* | + |
| 33 | +* | +* | + |
| 34 | +* | + | + |
| 35 | / | / | / |
| 36 | / | / | / |
| 37 | / | +* | + |
| 38 | / | / | / |
| 39 | + | + | + |

Continue.

| **Line** | ***Xbarc228*** | ***Xgdm6*** | ***Xwmc167*** |
|---|---|---|---|
| *Ae. kotschyi* | / | / | / |
| CS-S14 (hemi) | / | / | / |
| CS | + | + | + |
| W84-17 | +* | +* | + |
| 40 | / | / | / |
| 44 | + | + | + |
| 45 | / | / | / |
| 46 | / | / | / |
| 48 | +* | +* | + |
| 51 | +* | +* | + |
| 59 | +* | +* | + |
| 61 | / | / | / |
| 62 | +* | +* | + |
| 63 | +* | +* | + |
| 67 | / | / | / |
| 74 | / | +* | + |
| 77 | / | / | / |
| 78 | +* | +* | + |
| 79 | / | / | / |
| 80 | / | / | / |
| 81 | +* | +* | + |
| 83 | / | / | / |
| 84 | / | / | / |
| 85 | / | / | / |
| 86 | / | / | / |
| 89 | / | / | / |
| 90 | +* | +* | + |
| 91 | / | / | / |
| 92 | +* | +* | + |
| 93 | / | / | / |
| 94 | / | / | / |
| 97 | / | / | / |
| 98 | + | + | + |
| 99 | / | / | / |
| 103 | +* | +* | + |
| 104 | / | / | / |
| 106 | / | / | / |
| 107 | +* | +* | + |
| 108 | / | / | / |
| 109 | +* | +* | + |
| 110 | + | + | + |
| 115 | / | / | / |
| 116 | +* | +* | + |
| 117 | + | + | + |
| 119 | / | / | + |
| 124 | / | / | / |

Continue.

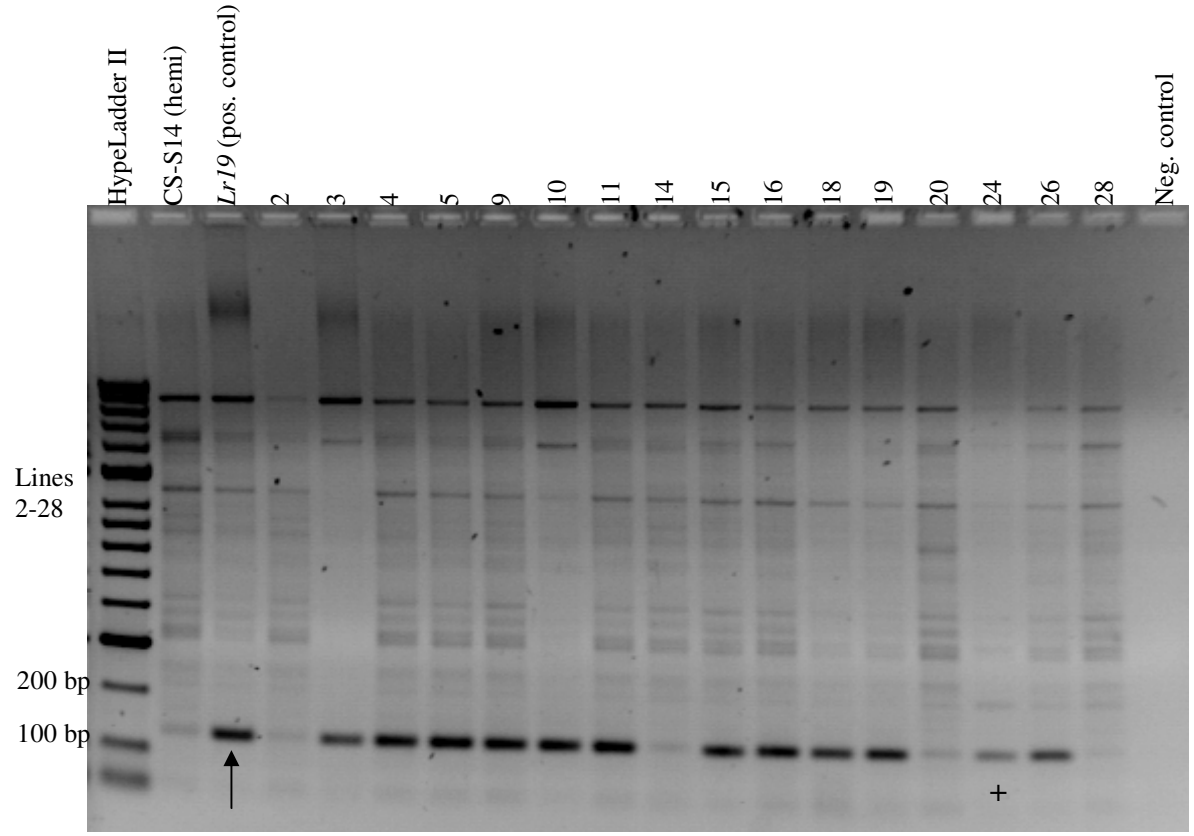
| **Line** | ***Xbarc228*** | ***Xgdm6*** | ***Xwmc167*** |
|---|---|---|---|
| *Ae. kotschyi* | / | / | / |
| CS-S14 (hemi) | / | / | / |
| CS | + | + | + |
| W84-17 | +* | +* | + |
| 125 | +* | +* | + |
| 128 | +* | +* | + |
| 129 | / | / | / |
| 130 | / | / | / |
| 131 | / | / | / |
| 139 | +* | +* | + |
| 143 | +* | +* | + |
| 148 | / | / | + |
| 156 | / | / | / |
| 159 | + | + | + |
| 165 | / | / | / |
| 174 | +* | +* | + |
| 178 | / | / | / |
| 180 | / | / | / |
| 181 | / | / | / |
| 182 | / | / | / |
| 184 | / | / | / |
| 185 | / | / | / |
| 187 | / | / | / |
| 192 | +* | +* | + |
| 195 | / | / | / |
| 196 | / | / | / |
| 197 | / | / | / |
| 198 | / | / | / |
| 200 | +* | +* | + |
| 201 | / | / | / |
| 202 | / | / | / |
| 204 | / | / | / |
| 205 | / | / | + |
| 206 | / | / | / |
| 207 | / | / | / |
| 210 | / | / | / |
| 213 | / | / | / |
| 214 | / | / | / |
| 215 | / | / | / |
| 217 | / | / | / |
| 219 | / | / | / |
| 220 | +* | +* | + |
| 221 | / | / | / |
| 225 | / | / | / |
| 226 | / | / | / |
| 227 | / | / | / |

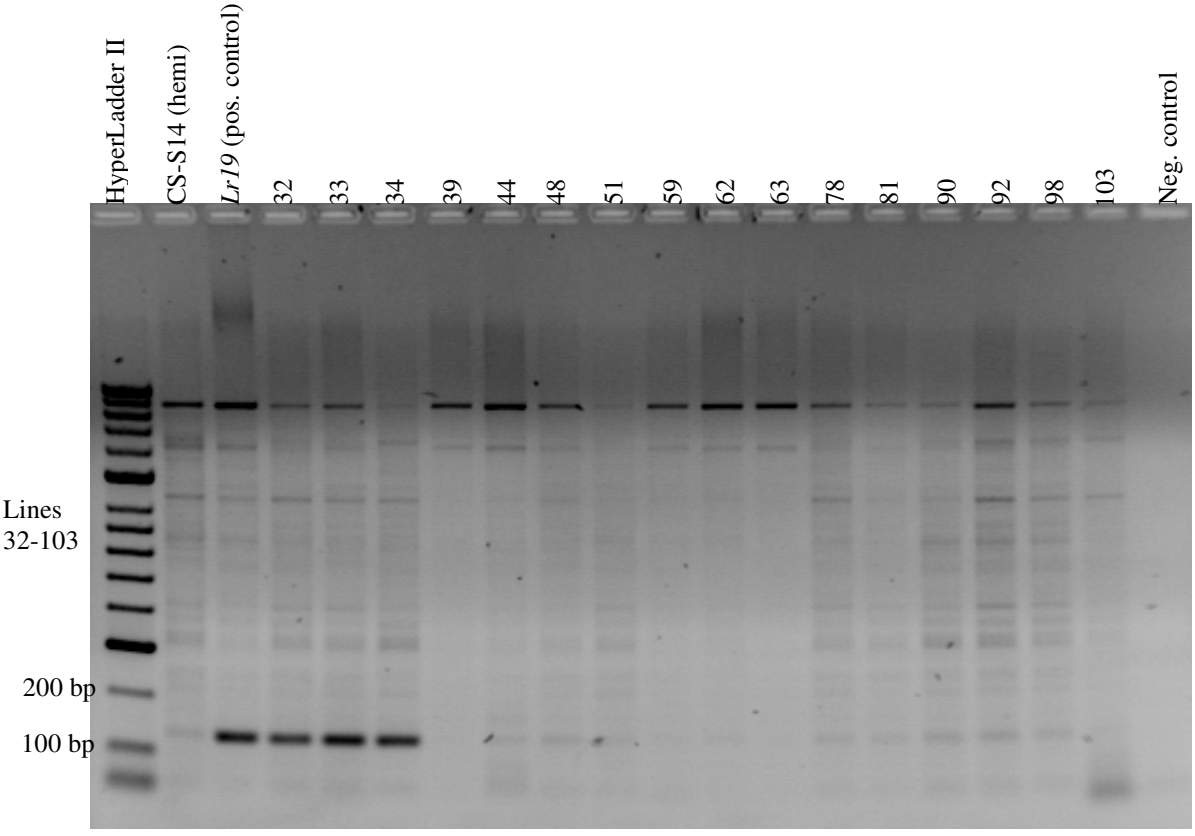
Continue.

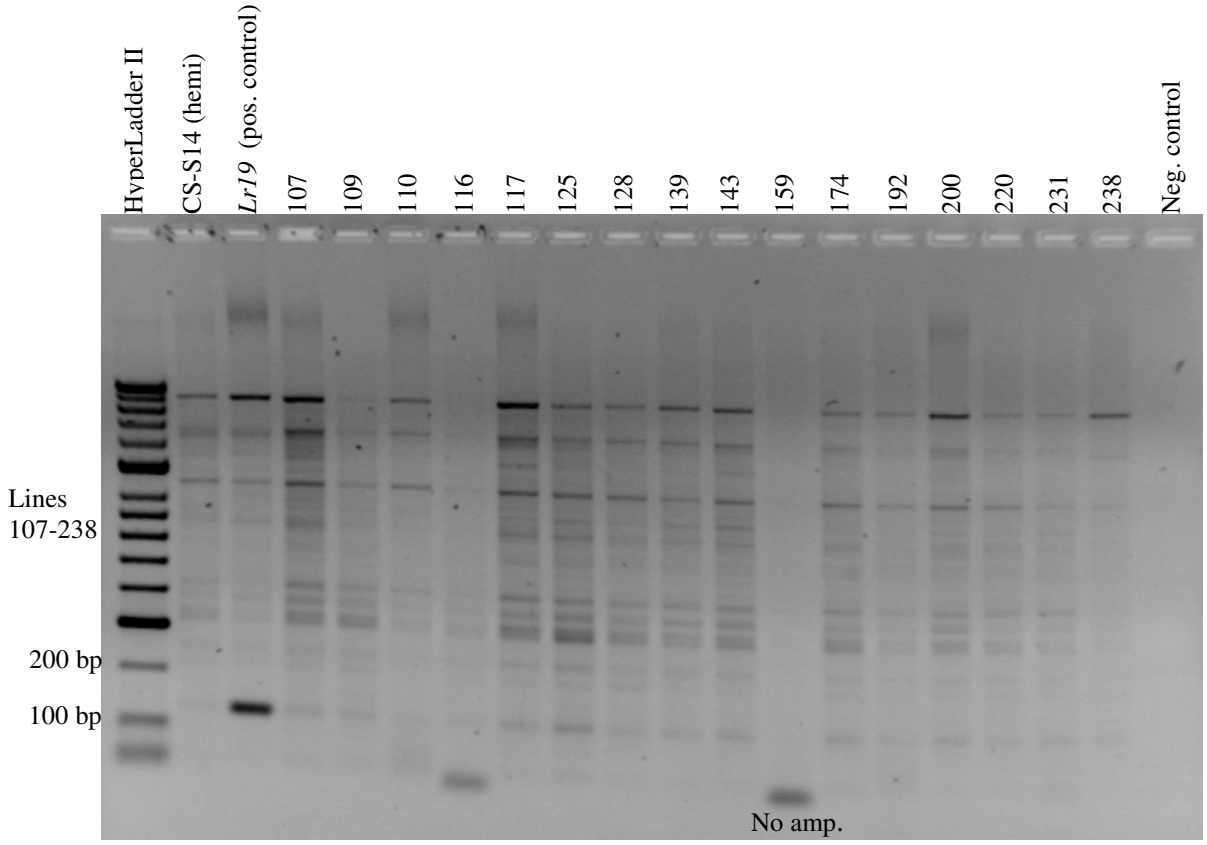
| **Line** | ***Xbarc228*** | ***Xgdm6*** | ***Xwmc167*** |
|---|---|---|---|
| *Ae. kotschyi* | / | / | / |
| CS-S14 (hemi) | / | / | / |
| CS | + | + | + |
| W84-17 | +* | +* | + |
| 228 | / | / | / |
| 229 | / | / | / |
| 230 | / | / | / |
| 231 | + | + | + |
| 232 | / | / | / |
| 233 | / | / | / |
| 234 | / | / | / |
| 236 | / | / | / |
| 237 | / | / | / |
| 238 | +* | +* | + |
| 239 | / | / | / |
| 240 | / | / | / |
| 241 | / | / | / |
| 242 | / | / | / |
| 243 | +* | +* | + |
| 244 | / | / | / |
| 245 | / | / | / |
| 246 | / | / | / |
| 247 | / | / | + |
| 248 | / | / | / |
| 249 | / | / | / |
| 250 | / | / | / |
| 252 | / | / | / |
| 254 | / | / | / |
| 255 | / | / | / |
| 256 | / | + | + |
| 257 | / | / | / |
| 258 | / | / | / |
| 259 | / | / | / |
| 260 | / | / | / |
| 261 | / | / | / |
| 262 | / | / | / |
| 263 | / | / | / |
| 264 | / | / | / |
| 265 | / | + | + |
| 266 | / | / | / |
| 267 | / | / | / |
| 269 | / | / | / |
| 272 | / | / | / |
| 273 | / | / | + |
| 274 | +* | +* | + |
| 275 | / | / | / |
| 276 | / | / | / |

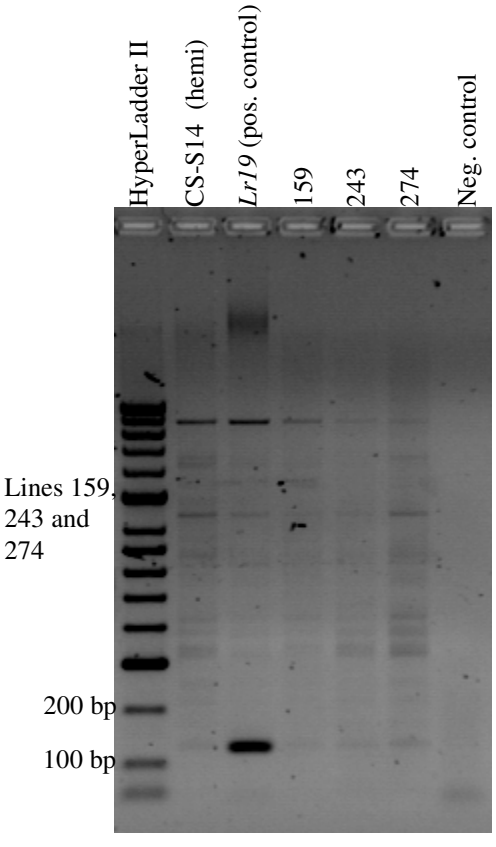
# Addendum B

Lines that appeared to be recombinants were screened for the presence of *Lr19* using an STS marker, *STSLr19*$_{130}$. The arrow indicates the 130 bp band amplified in lines with *Lr19*.



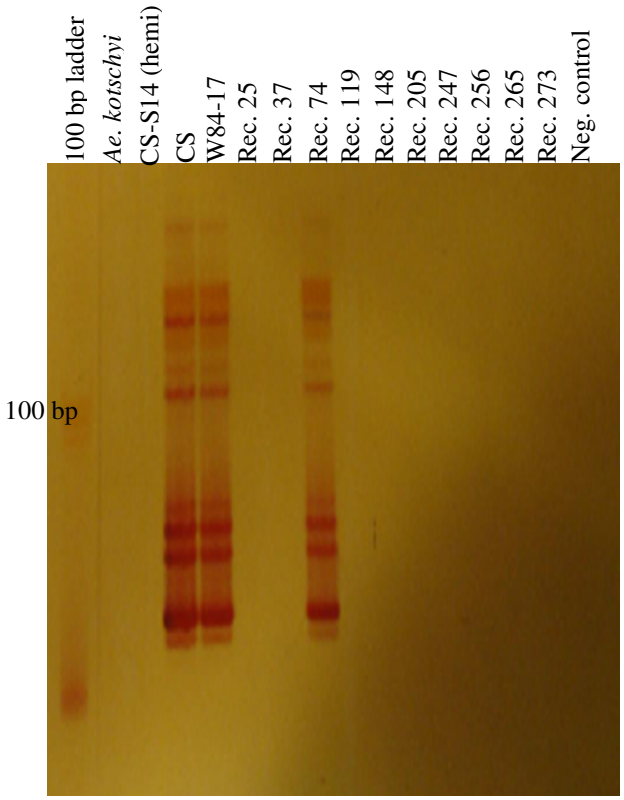




HyperLadder II
CS-S14 (hemi)
Lr19 (pos. control)
107
109
110
116
117
125
128
139
143
159
174
192
200
220
231
238
Neg. control
Lines 107-238
200 bp
100 bp
No amp.

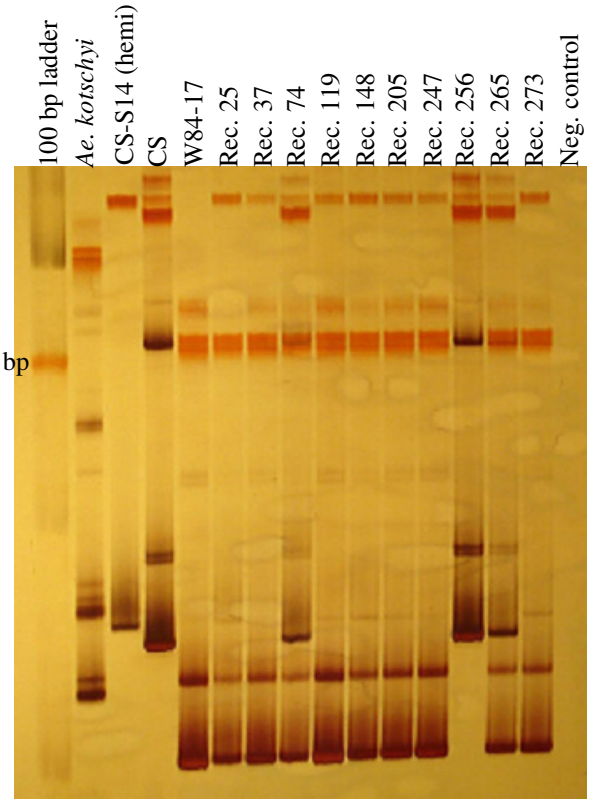

HyperLadder II
CS-S14 (hemi)
Lr19 (pos. control)
159
243
274
Neg. control
Lines 159, 243 and 274
200 bp
100 bp

# Addendum C

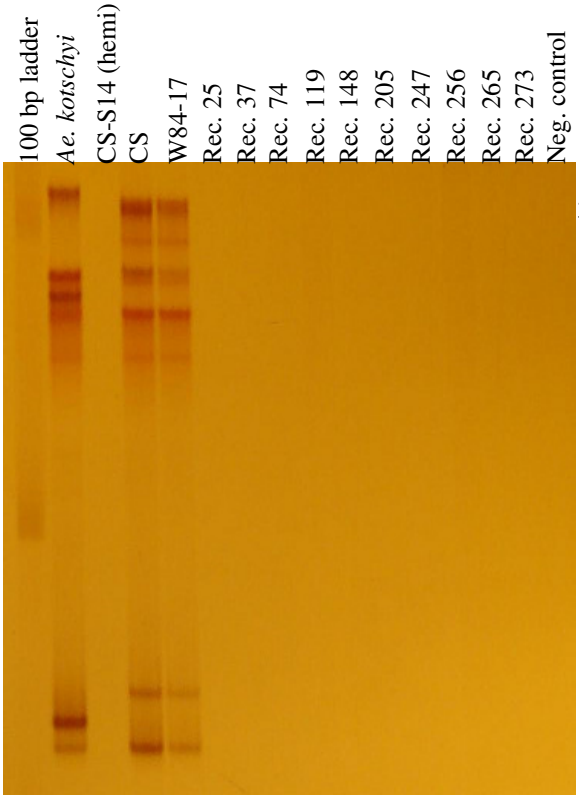
Amplification products of microsatellite markers used to identify and characterize translocation recombinants.



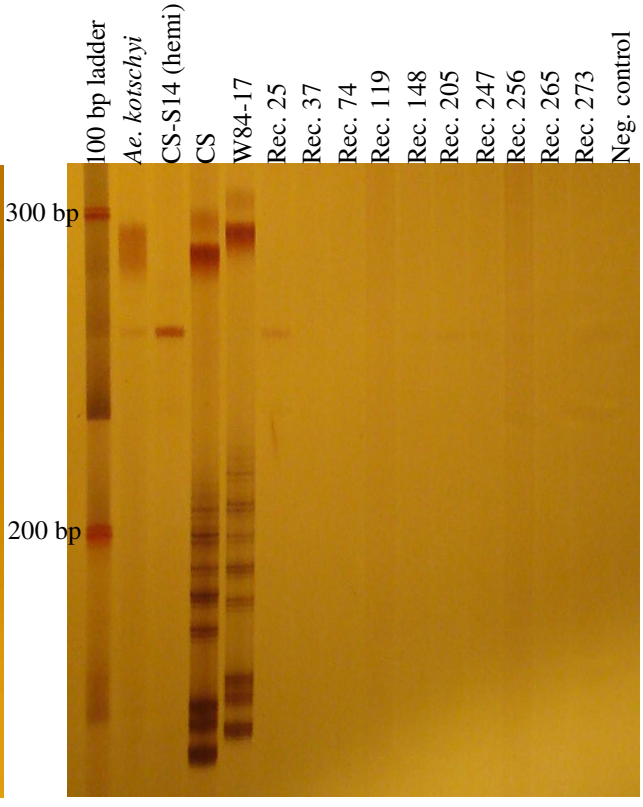

**Gwm157 (2DL)**



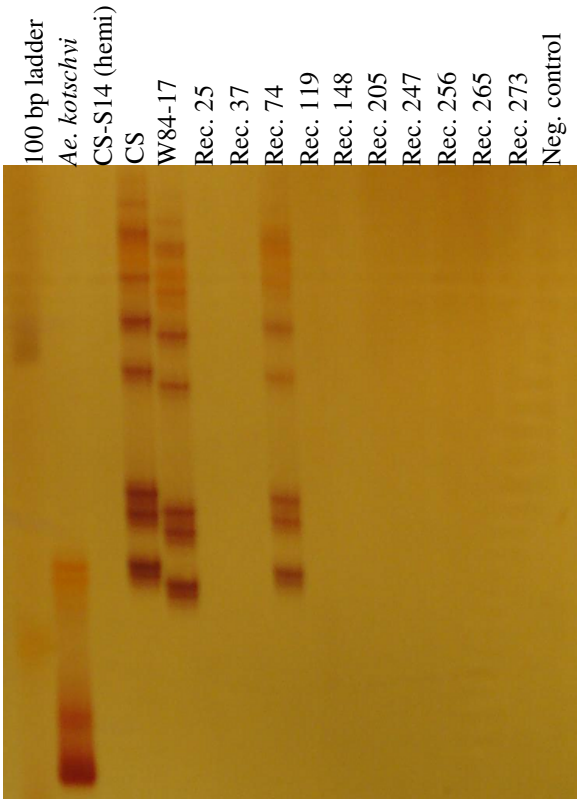

**Cfd50 (2DL)**



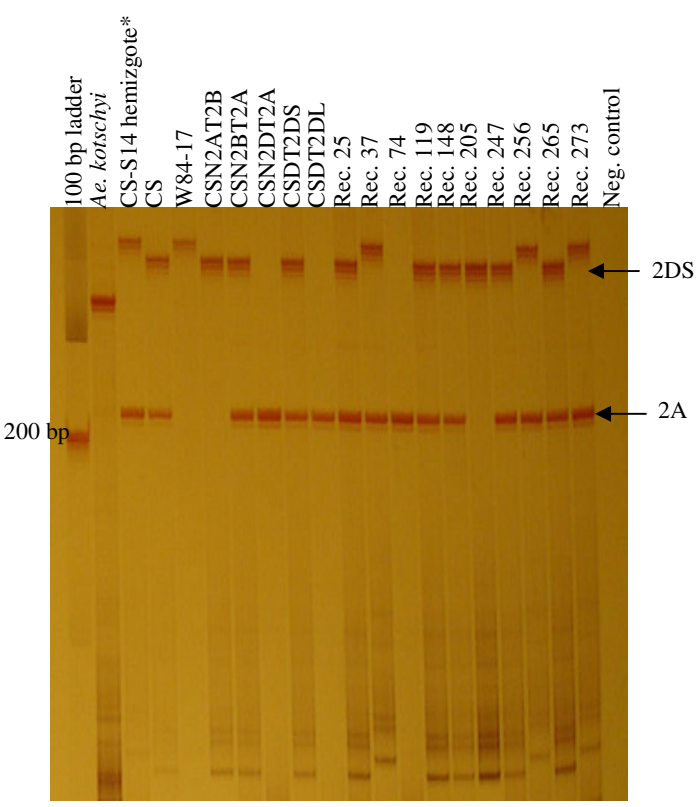

**Wmc41 (2DL)**



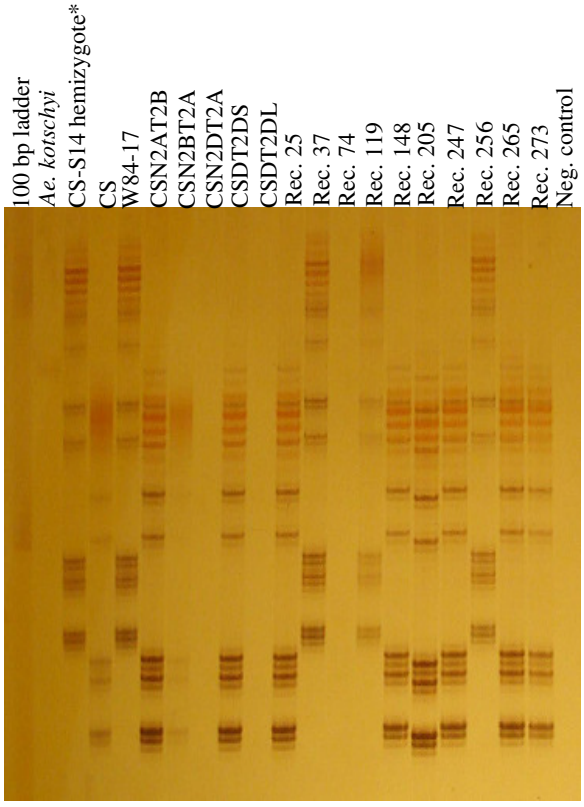

**Cfd233 (2DL)**



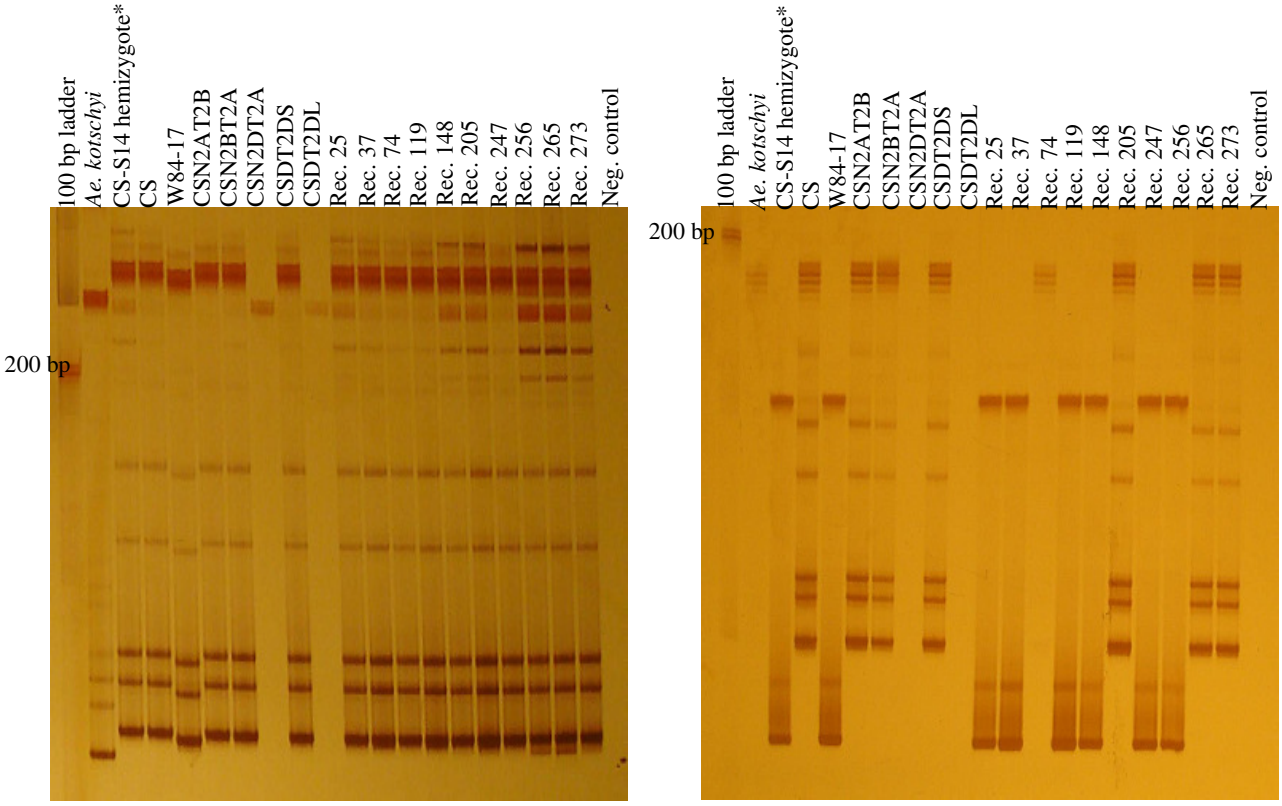

**Gwm539 (2DL)**




**Barc124 (2DS and 2A)**




**Gwm484 (2DS)**

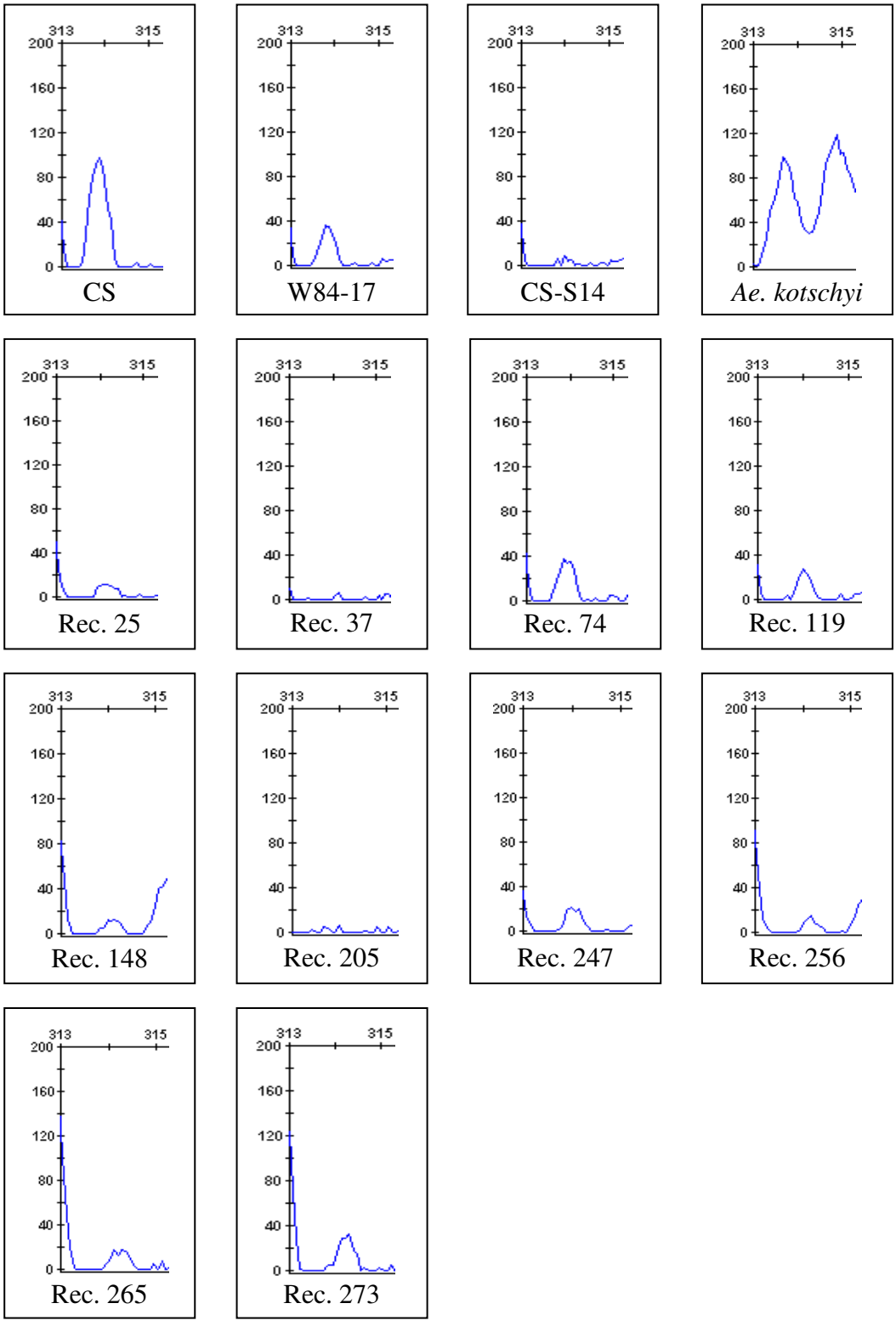



**Cfd116 (2DS)** **Gwm261 (2DS)**

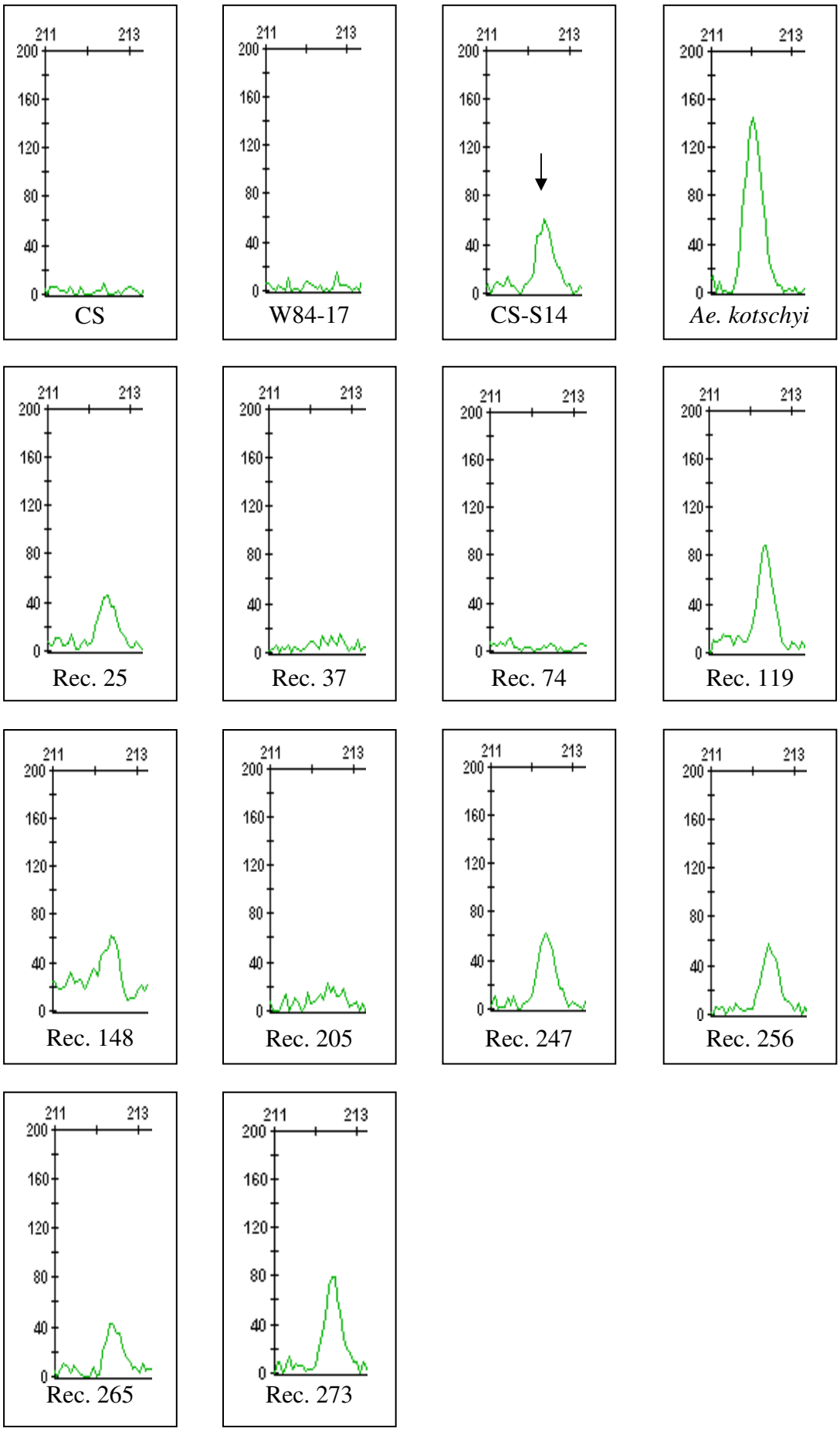
*04M144-1 ($F_2$) hemizygous for translocation

# Addendum D

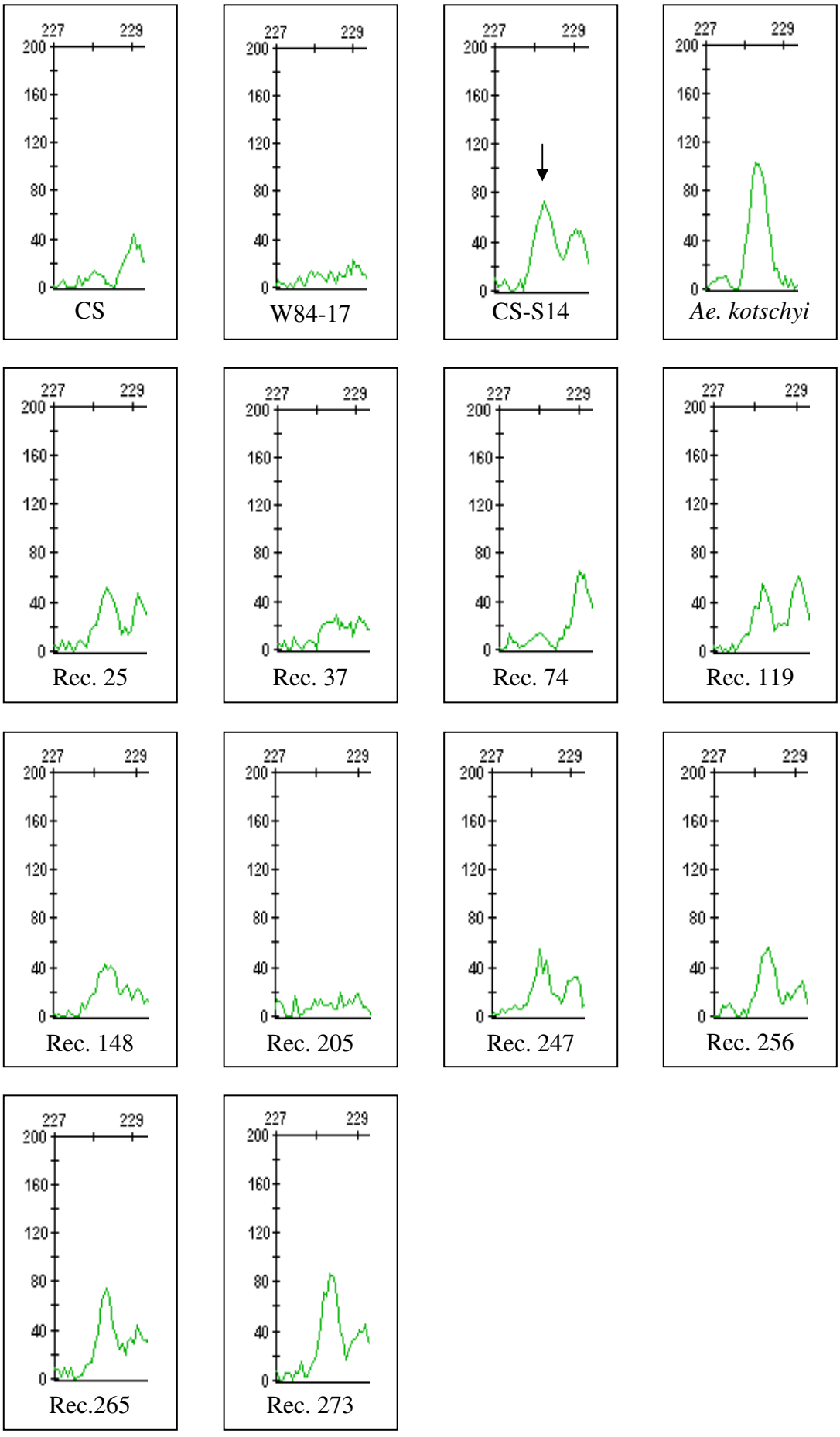
AFLP bands that were found to be associated with the S14 translocation. For selective amplification, *EcoRI* primers labeled with different fluorescent dyes (*Eco*RI – ACA (FAM blue), *Eco*RI – AAC (NED yellow) and *Eco*RI – AGG (JOE green)) were used in combination with *Mse*I selective primers (CAG, CTG, CAT, CTC and CTA).



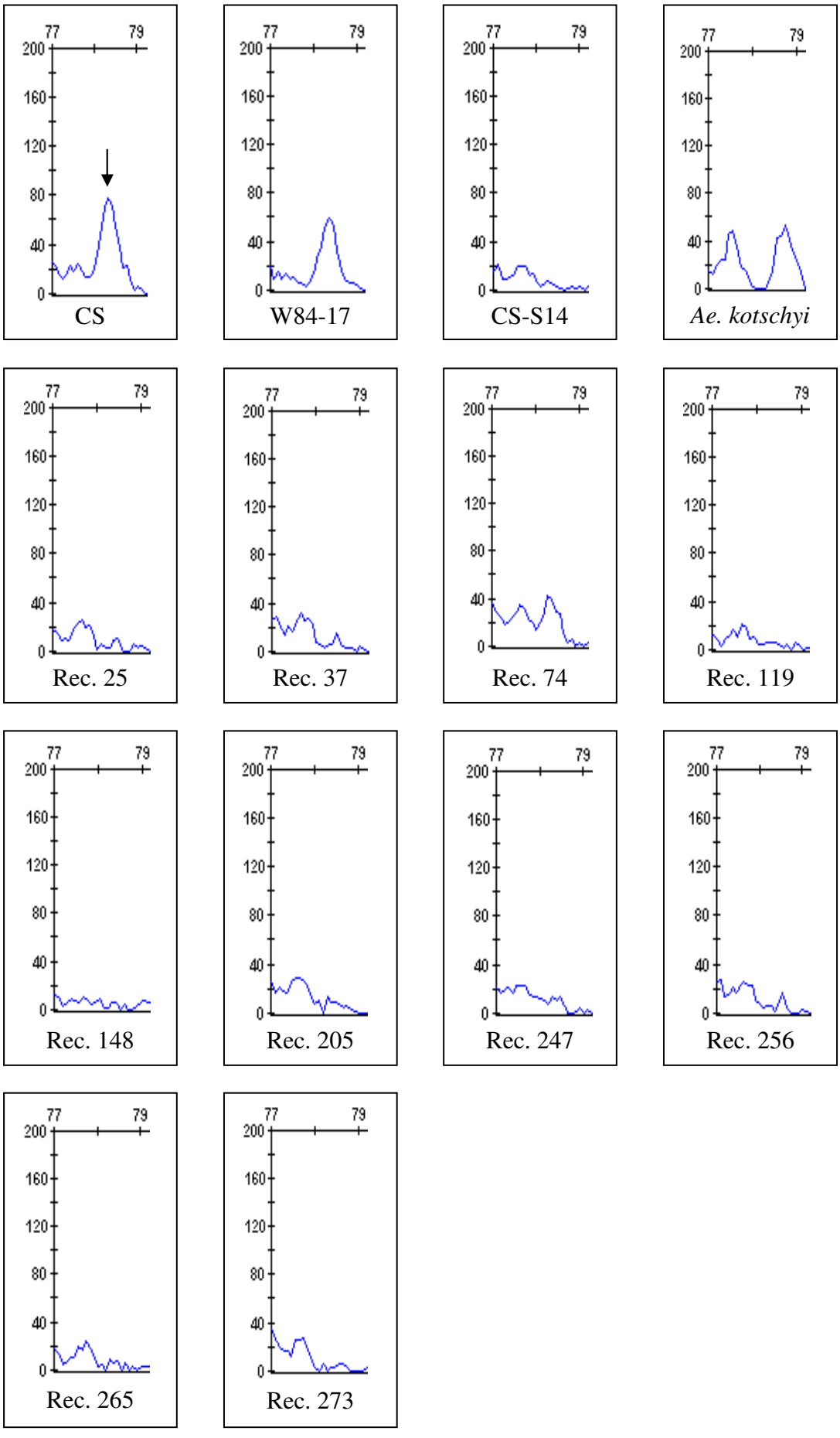

AFLP primer combination *Eco*RI - ACA/*Mse*I - CAG - polymorphic allele size 314 bp



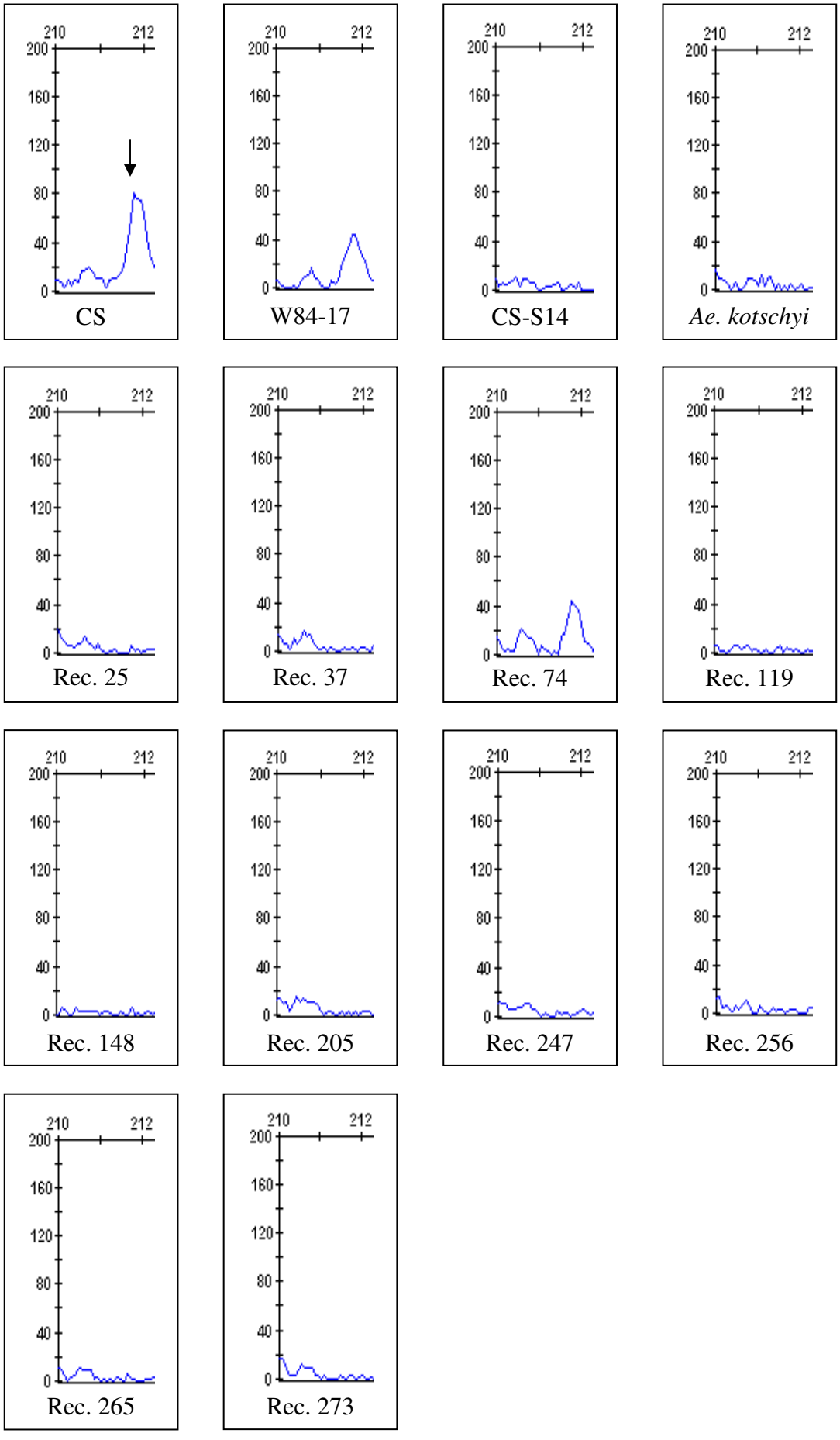

AFLP primer combination *Eco*RI - AGG/*Mse*I - CAG - polymorphic allele size 212 bp



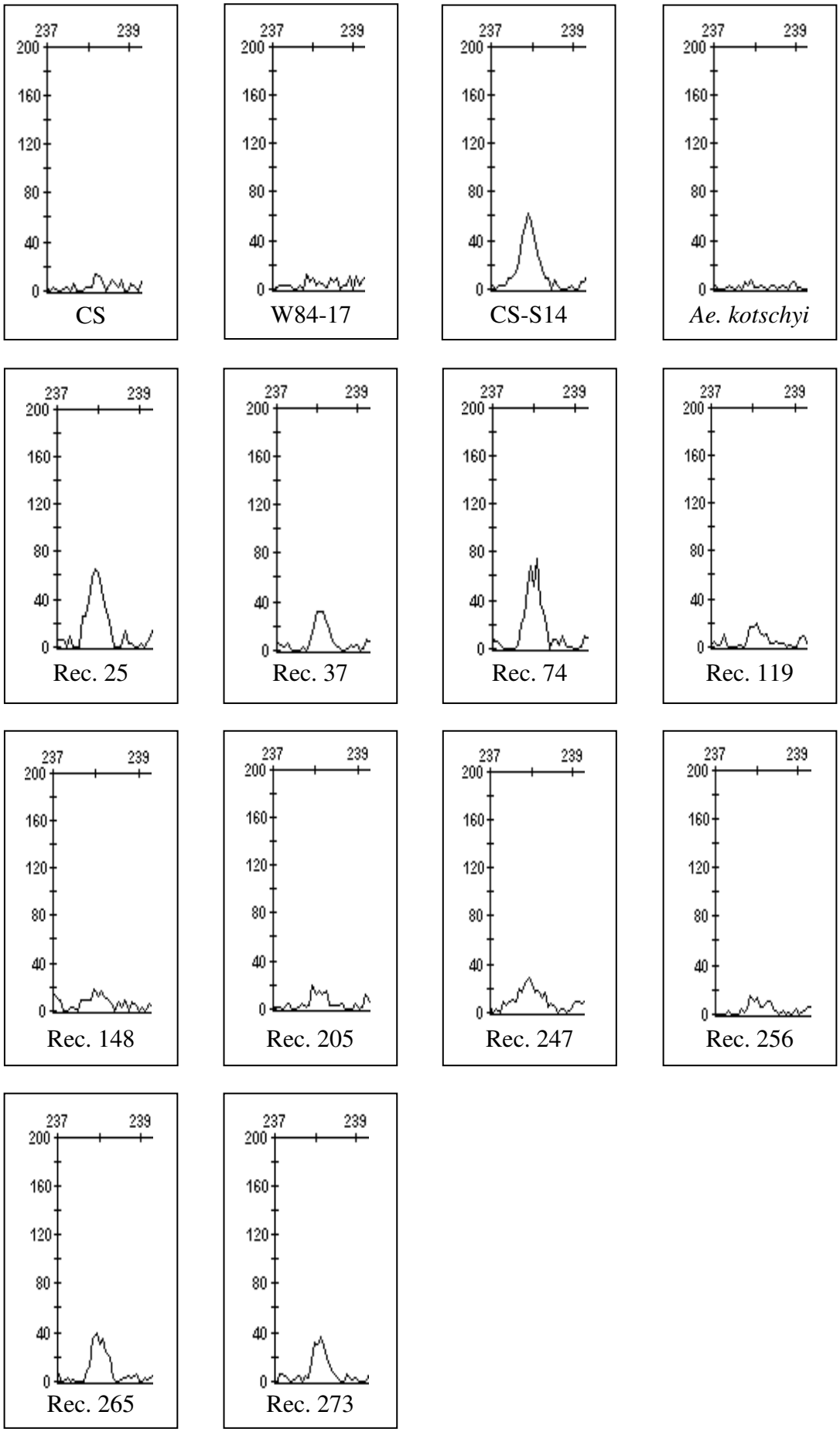

AFLP primer combination *Eco*RI - AGG/*Mse*I - CAG - polymorphic allele size 228 bp



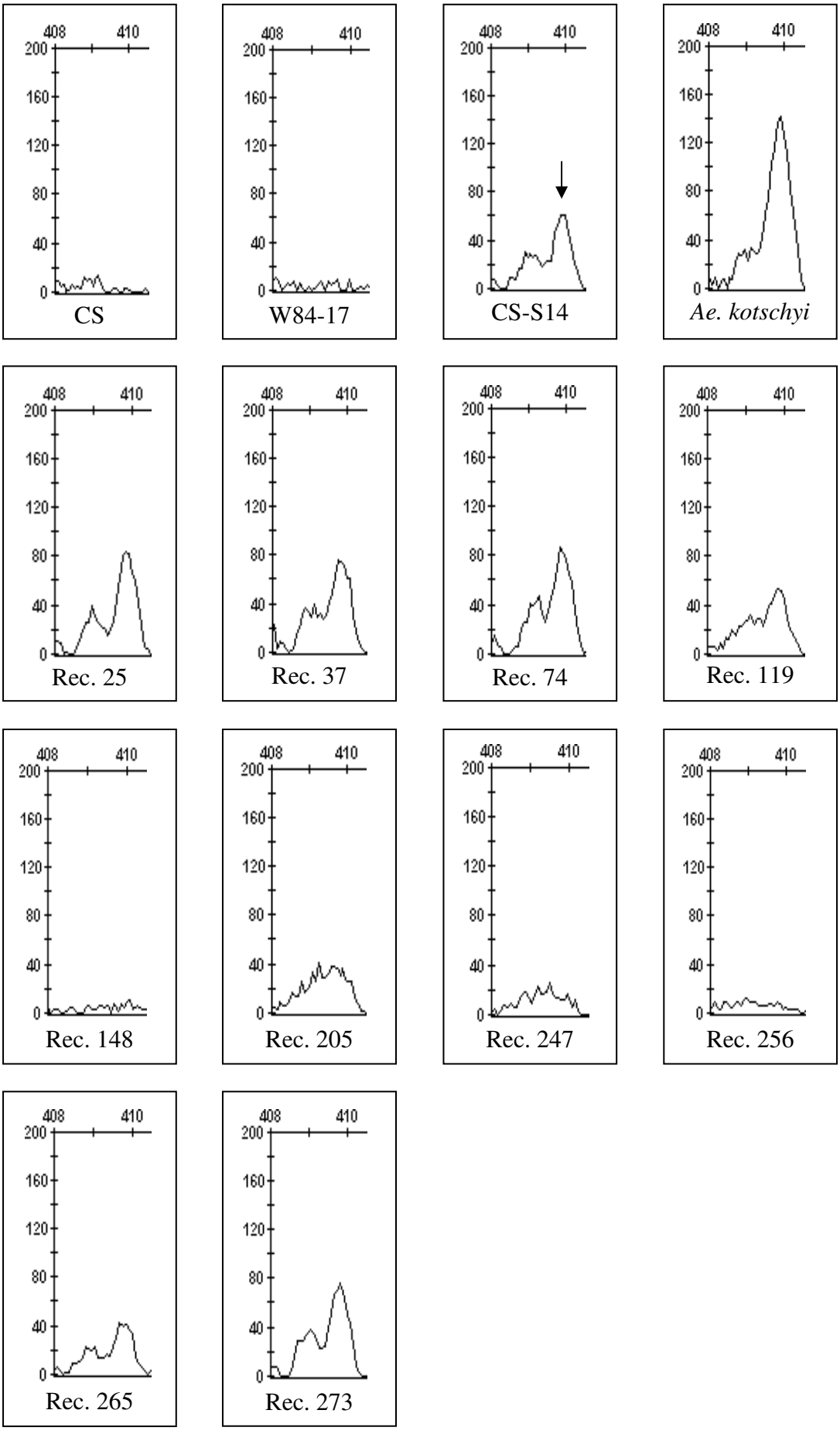

AFLP primer combination *Eco*RI - ACA/*Mse*I - CAT - polymorphic allele size 78 bp



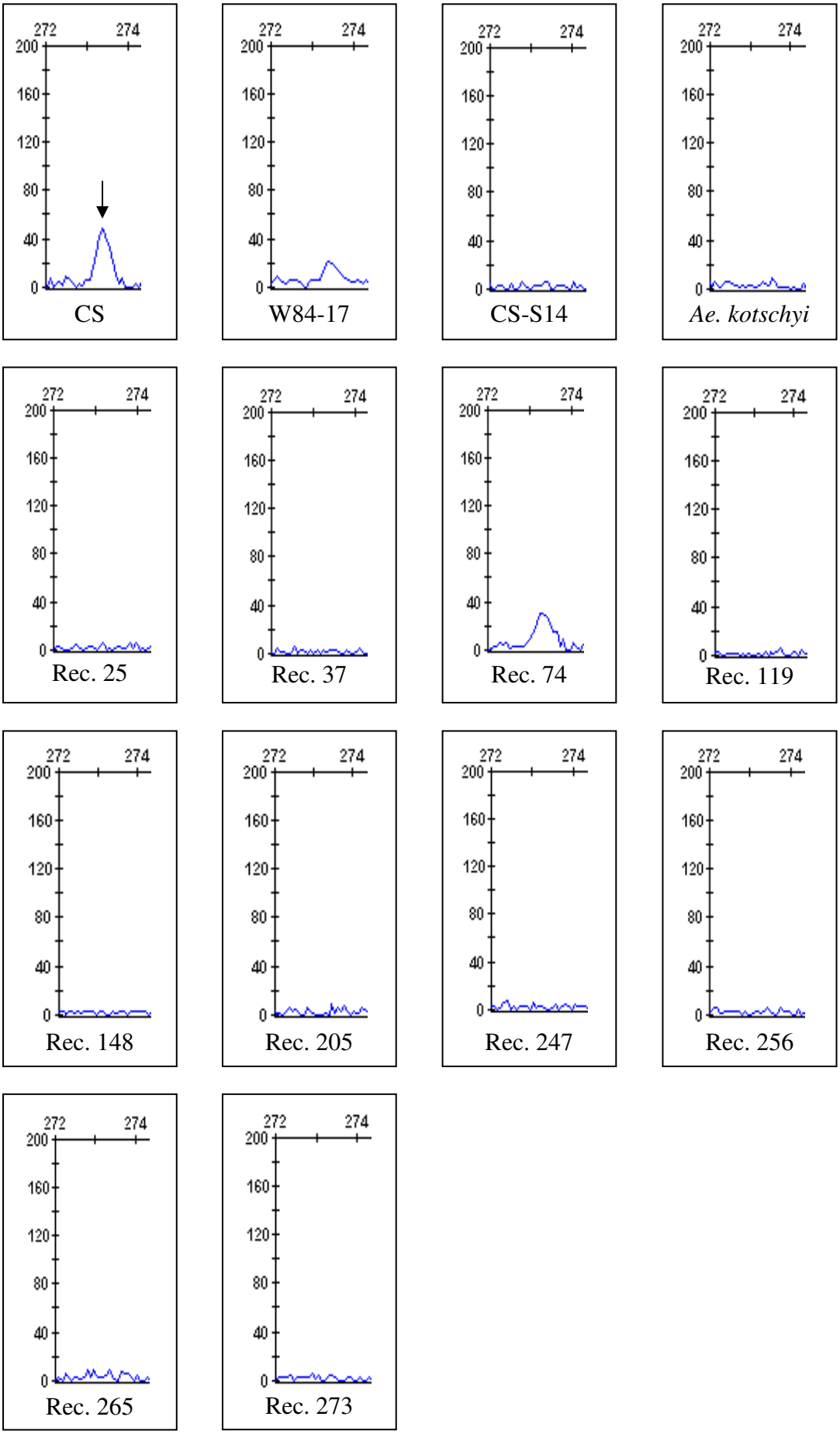

AFLP primer combination *Eco*RI - ACA/*Mse*I - CAT - polymorphic allele size 212 bp



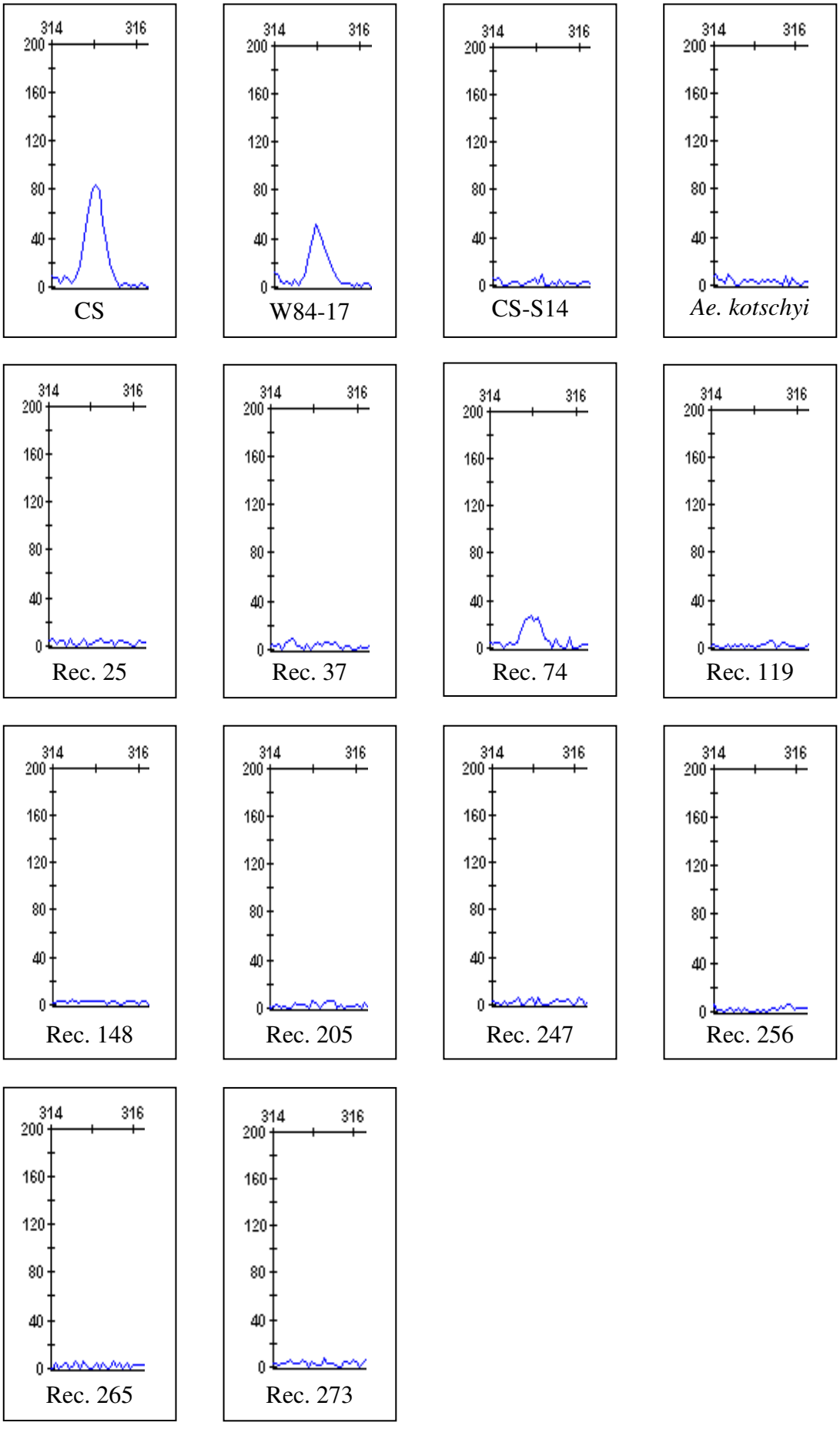

AFLP primer combination *Eco*RI - AAC/*Mse*I - CAT - polymorphic allele size 238 bp



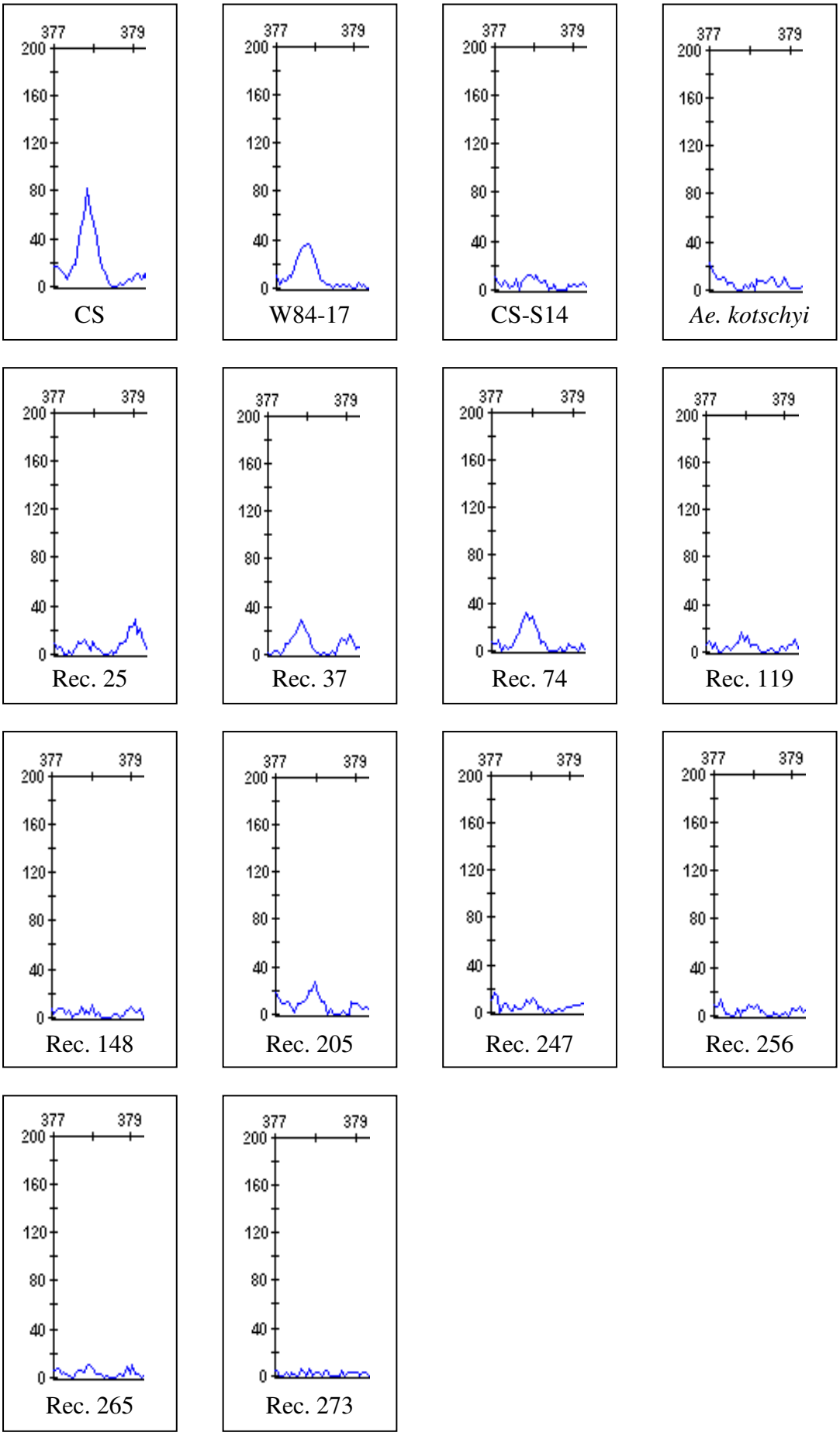

AFLP primer combination *Eco*RI - AAC/*Mse*I - CAT - polymorphic allele size 410 bp



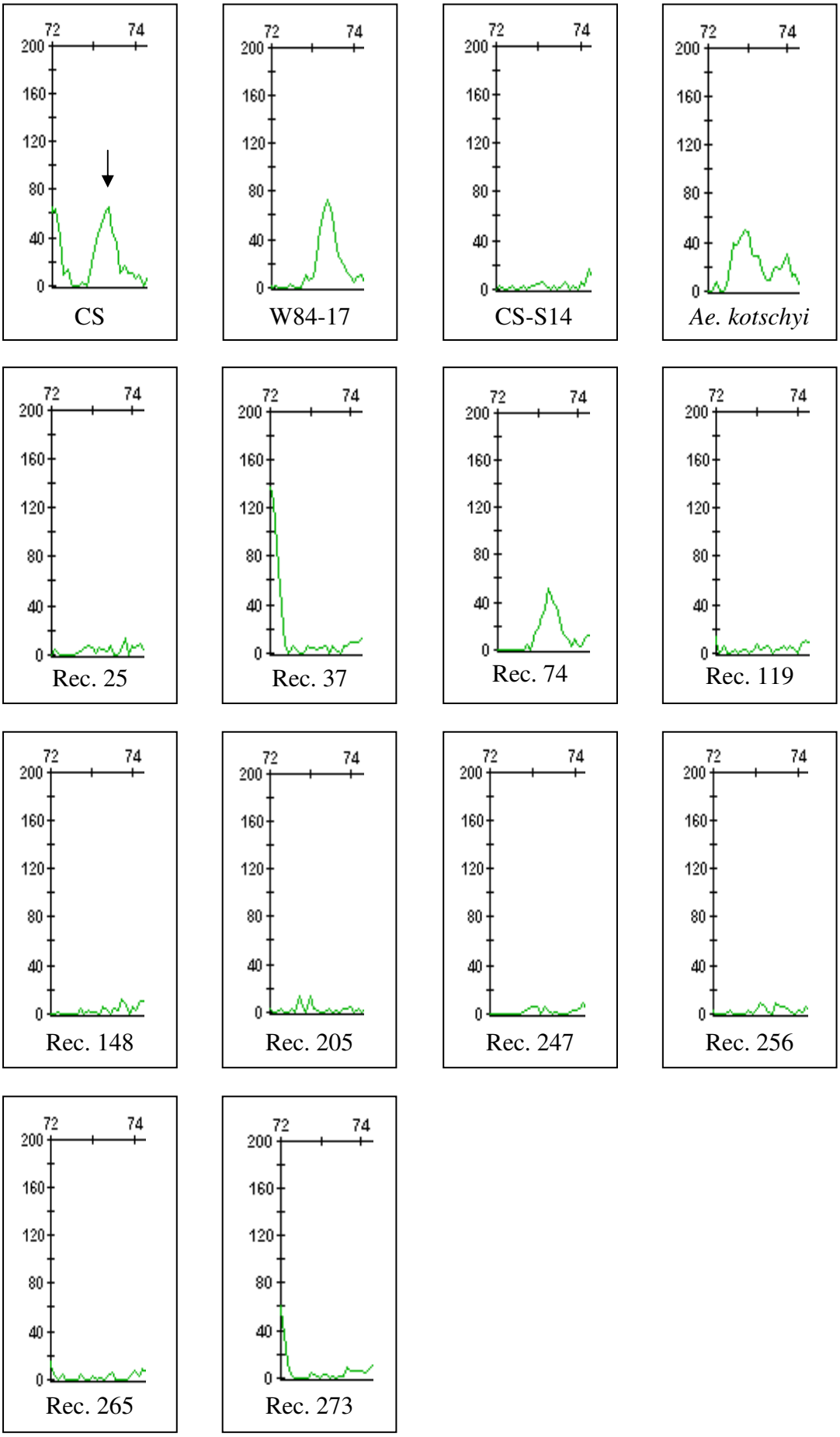

AFLP primer combination *Eco*RI - ACA/*Mse*I - CTA - polymorphic allele size 273 bp



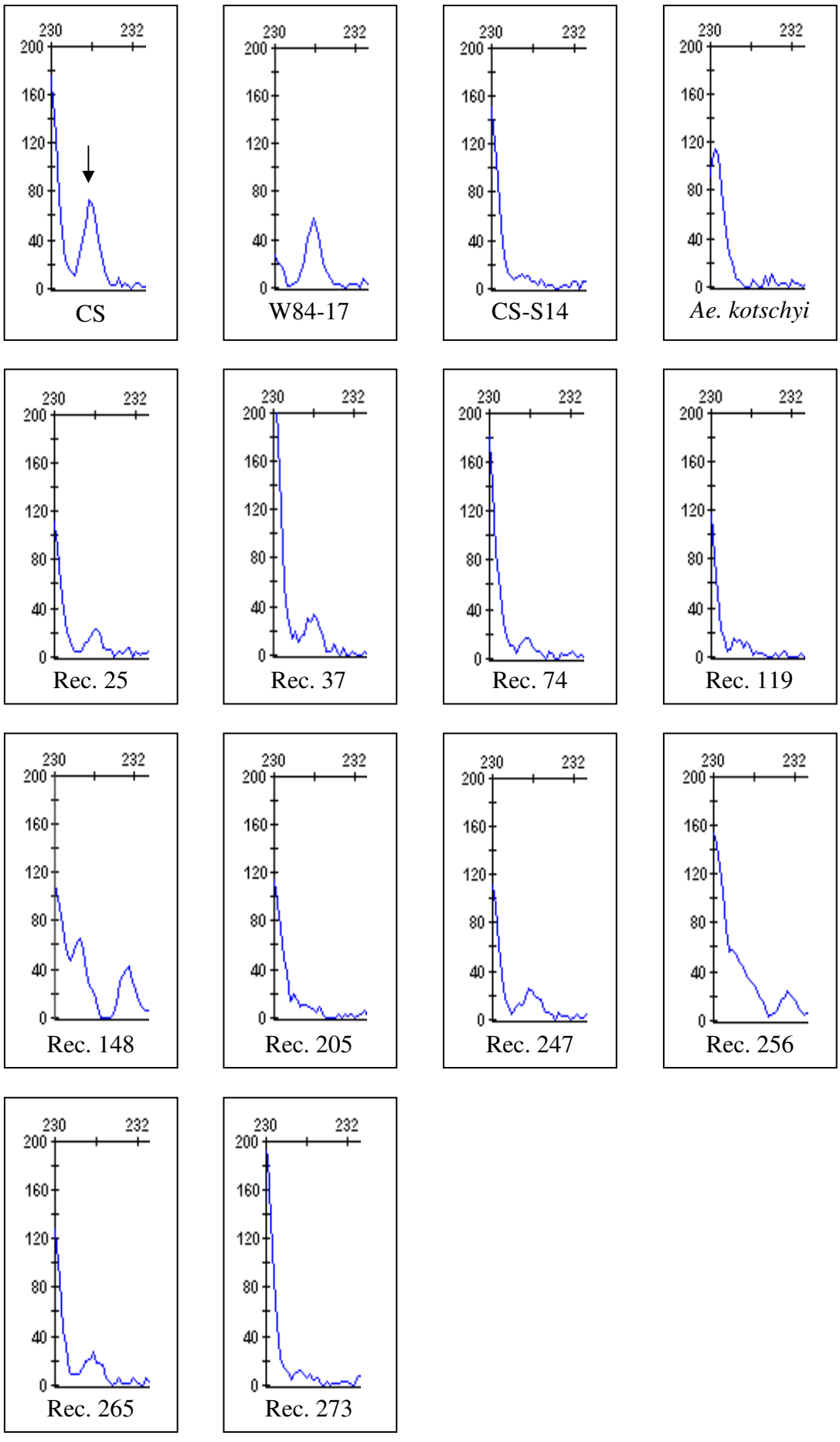

AFLP primer combination *Eco*RI - ACA/*Mse*I - CTA - polymorphic allele size 315 bp



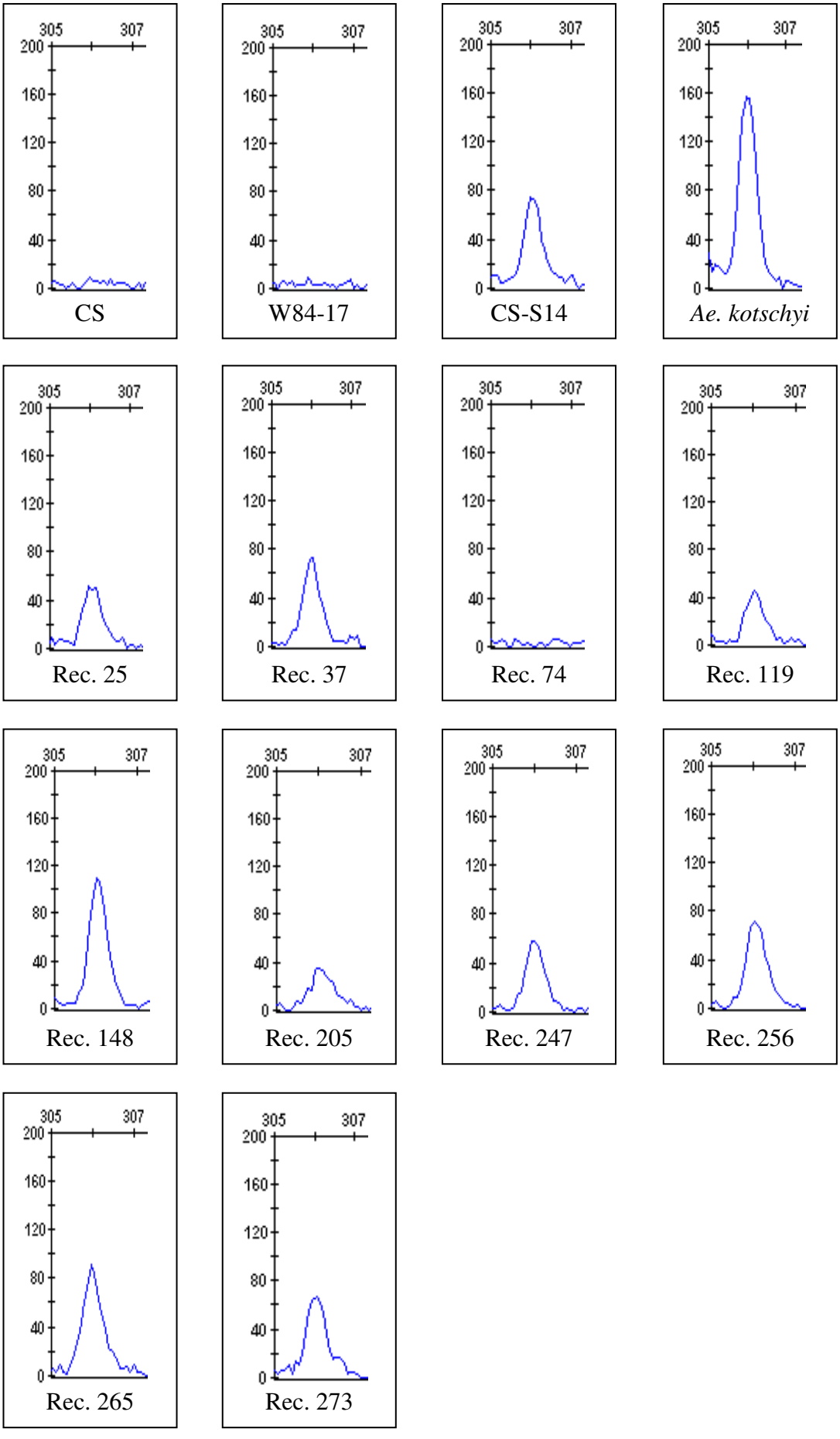

AFLP primer combination *Eco*RI - ACA/*Mse*I - CTA - polymorphic allele size 378 bp



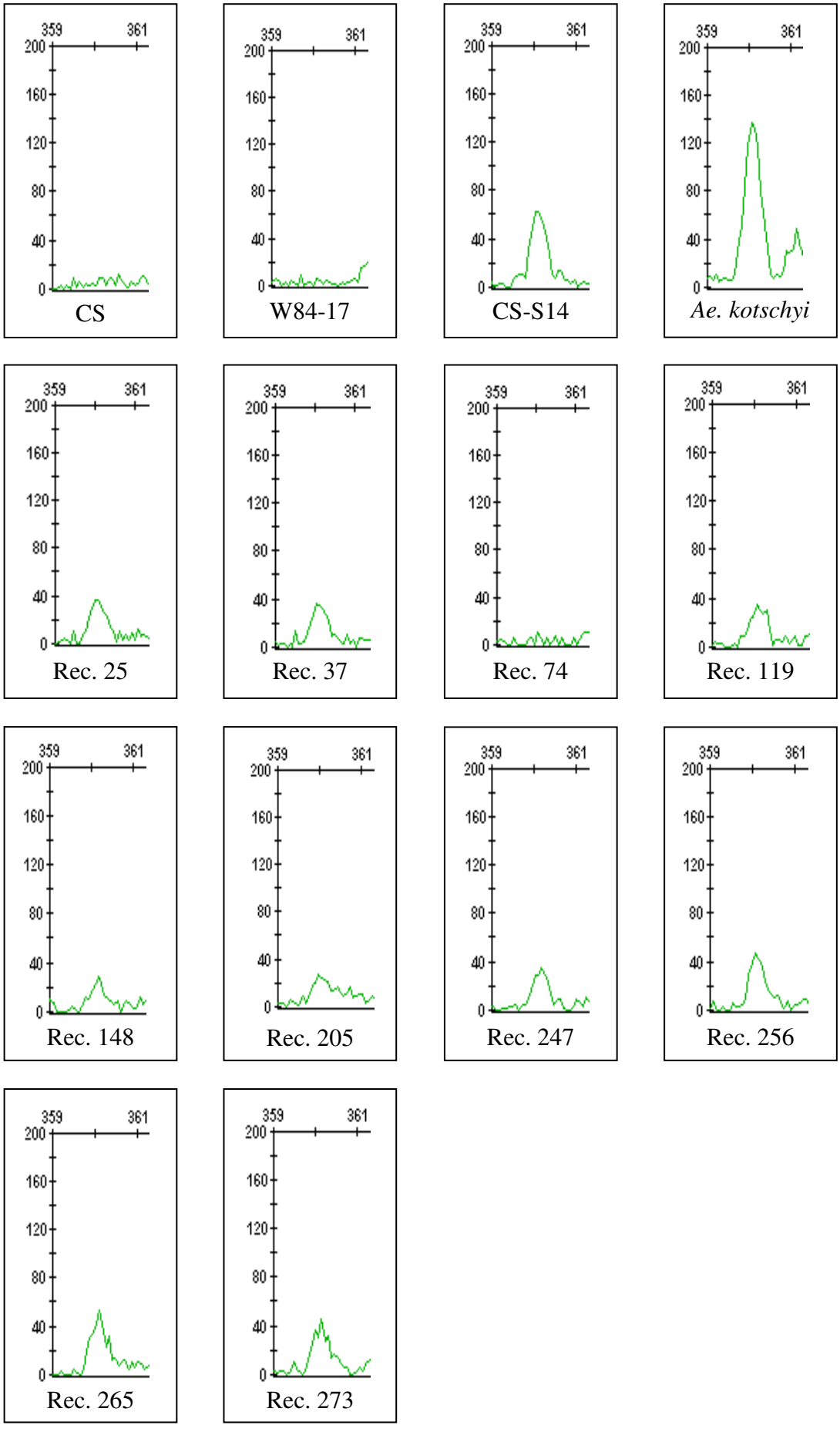

AFLP primer combination *Eco*RI - AGG/*Mse*I - CTA - polymorphic allele size 73 bp



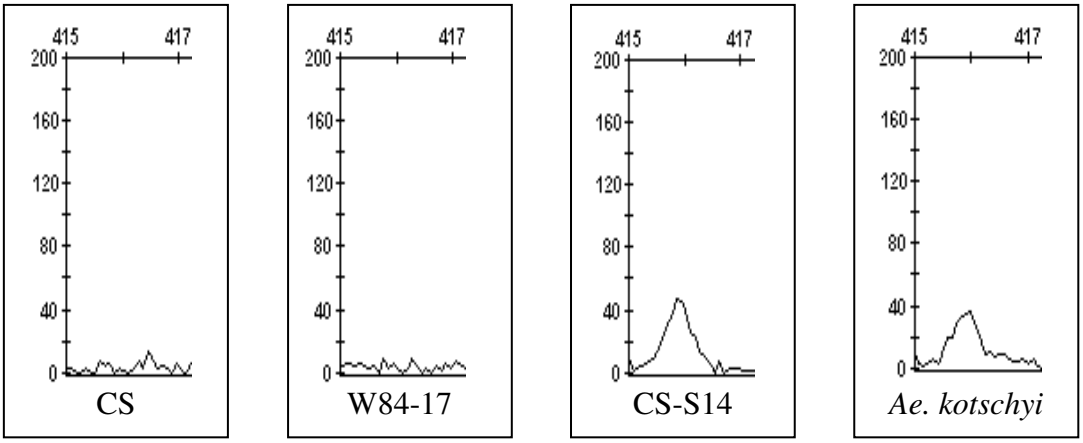

AFLP primer combination *Eco*RI - ACA/*Mse*I - CTG - polymorphic allele size 231 bp

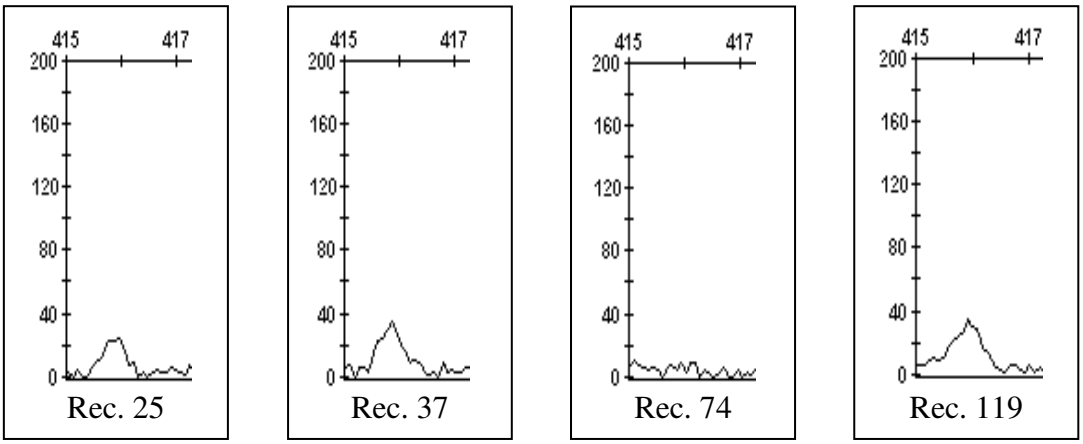


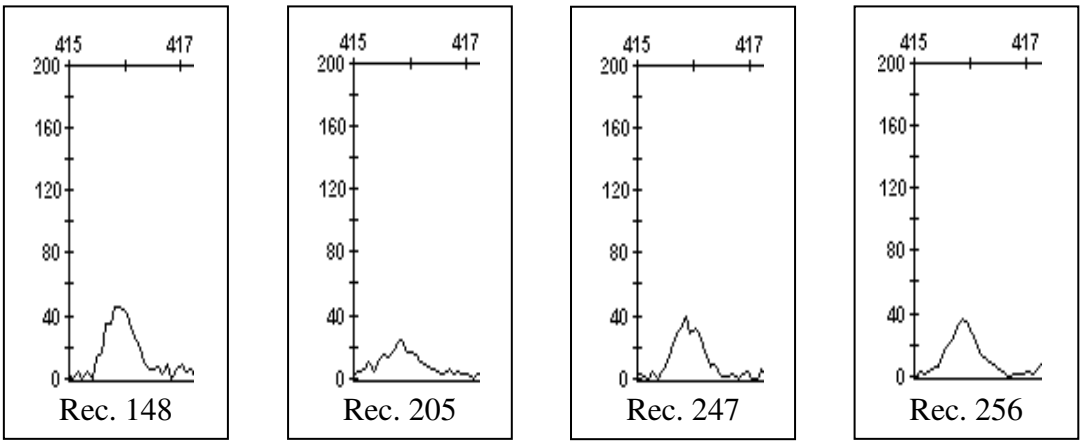

AFLP primer combination *Eco*RI - ACA/*Mse*I - CTG - polymorphic allele size 306 bp

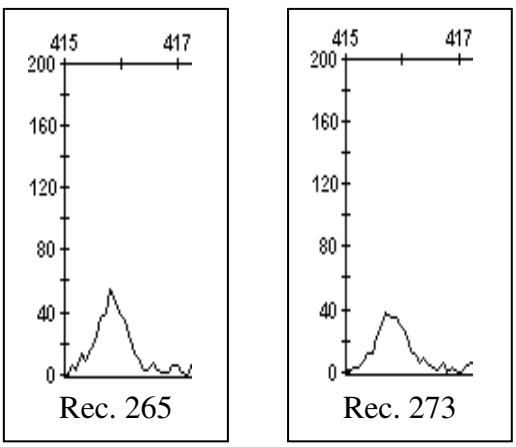



AFLP primer combination *Eco*RI - AGG/*Mse*I - CTG - polymorphic allele size 360 bp



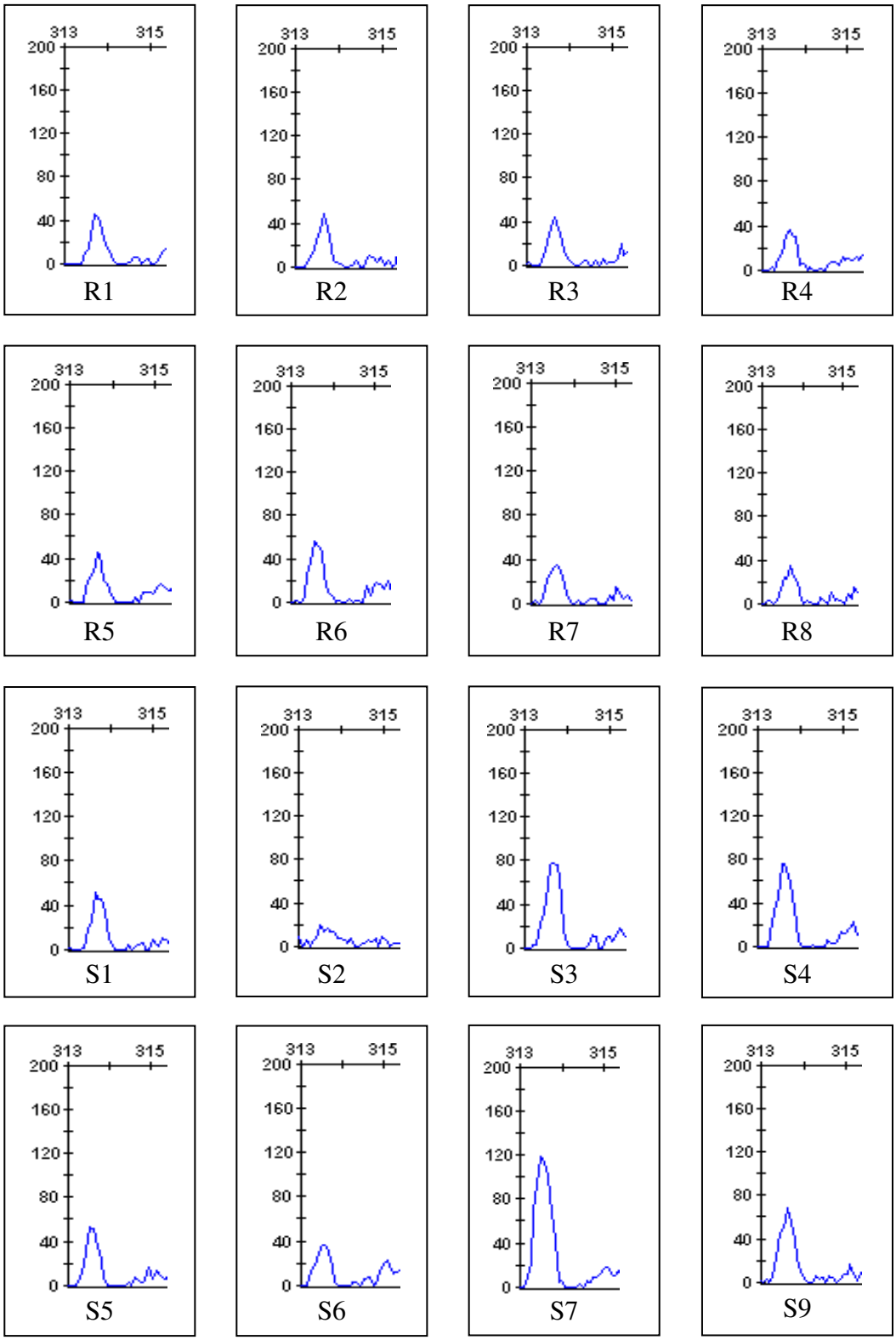



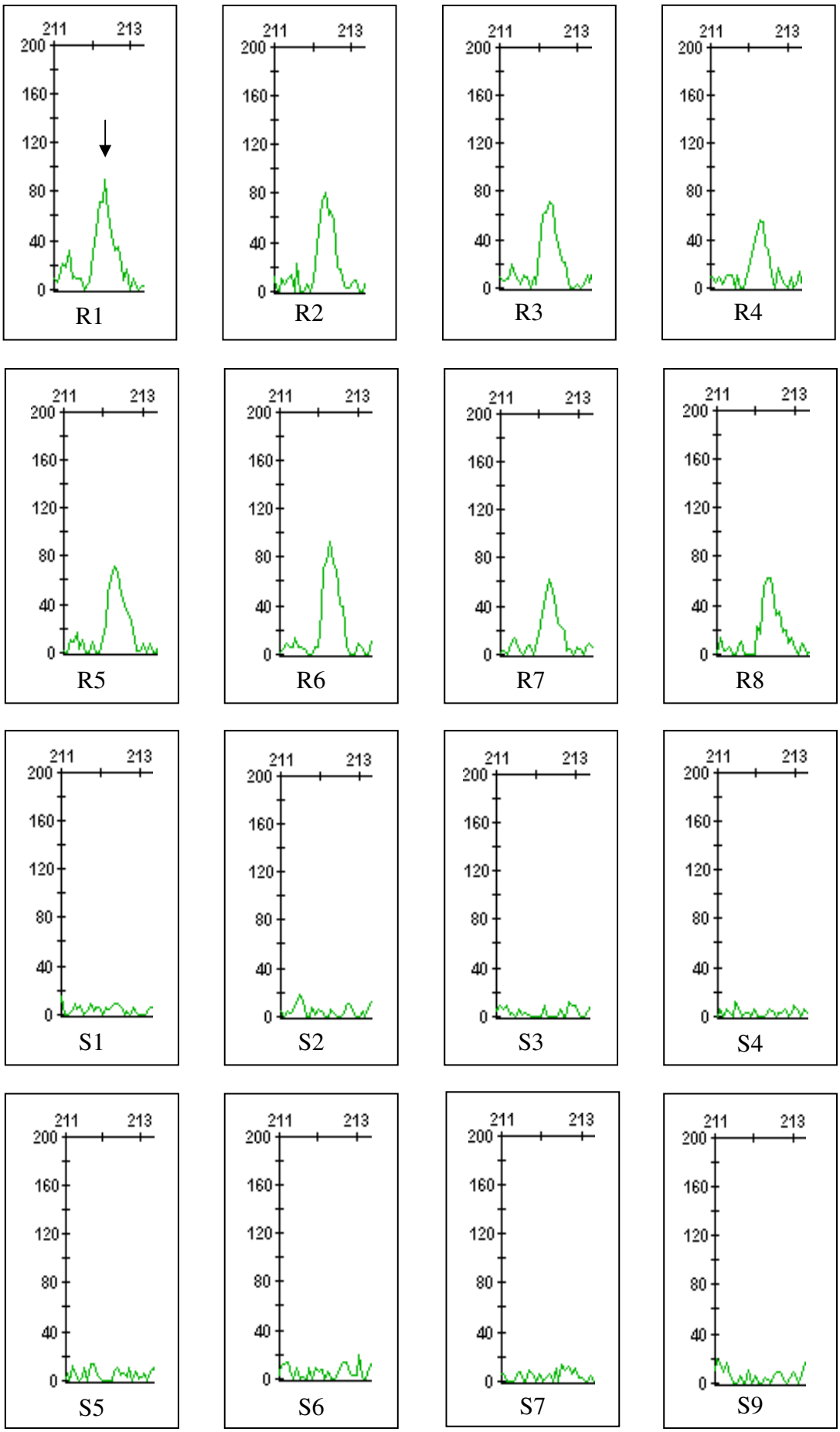

AFLP primer combination *Eco*RI - AAC/*Mse*I - CTG - polymorphic allele size 416 bp

# Addendum E

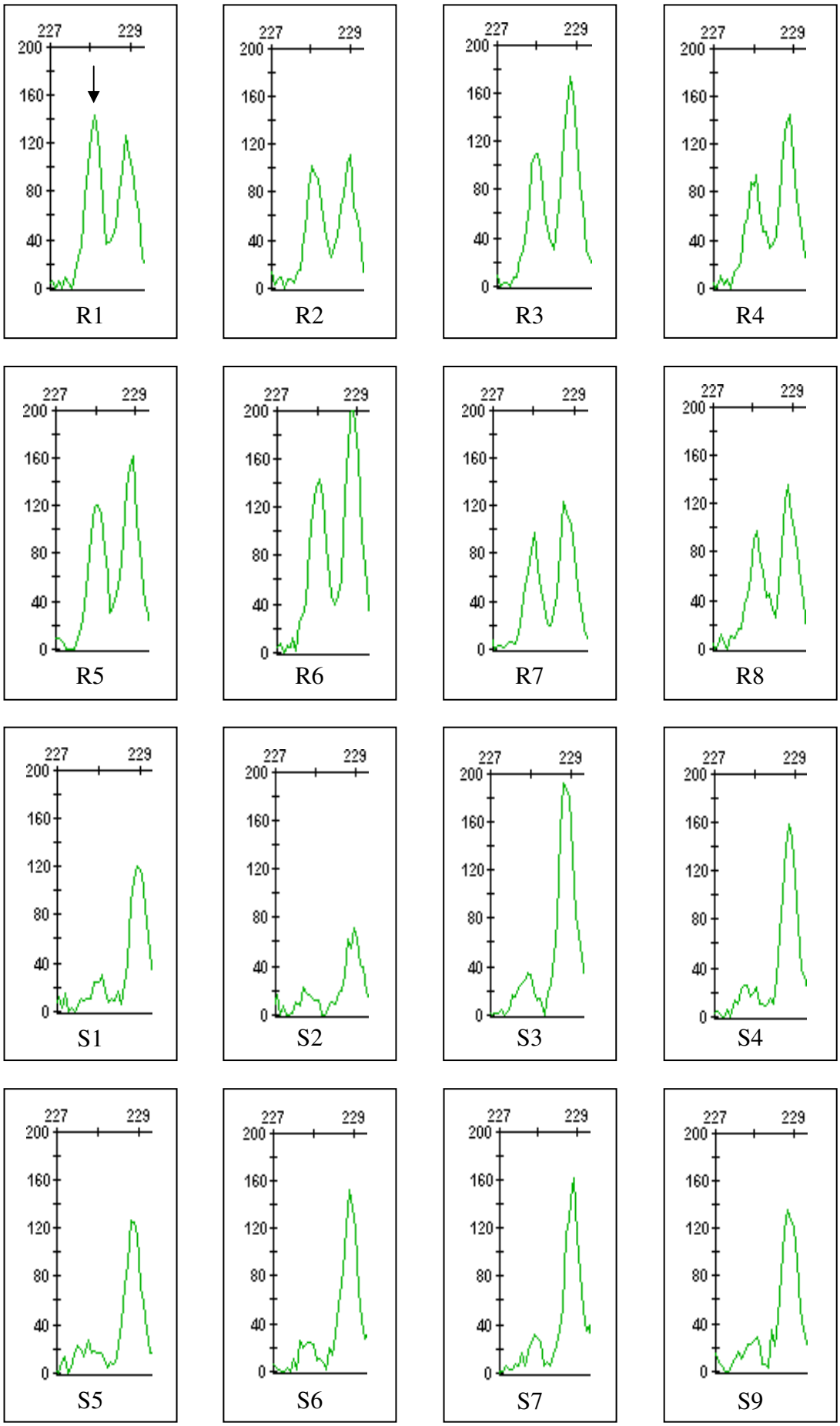
Confirmation of polymorphic AFLP loci and its association with the S14 translocation in a panel of 16 plants with (resistant) and without (susceptible) the S14 translocation (cross = CS-S14 translocation/2*CS*ph1b*).



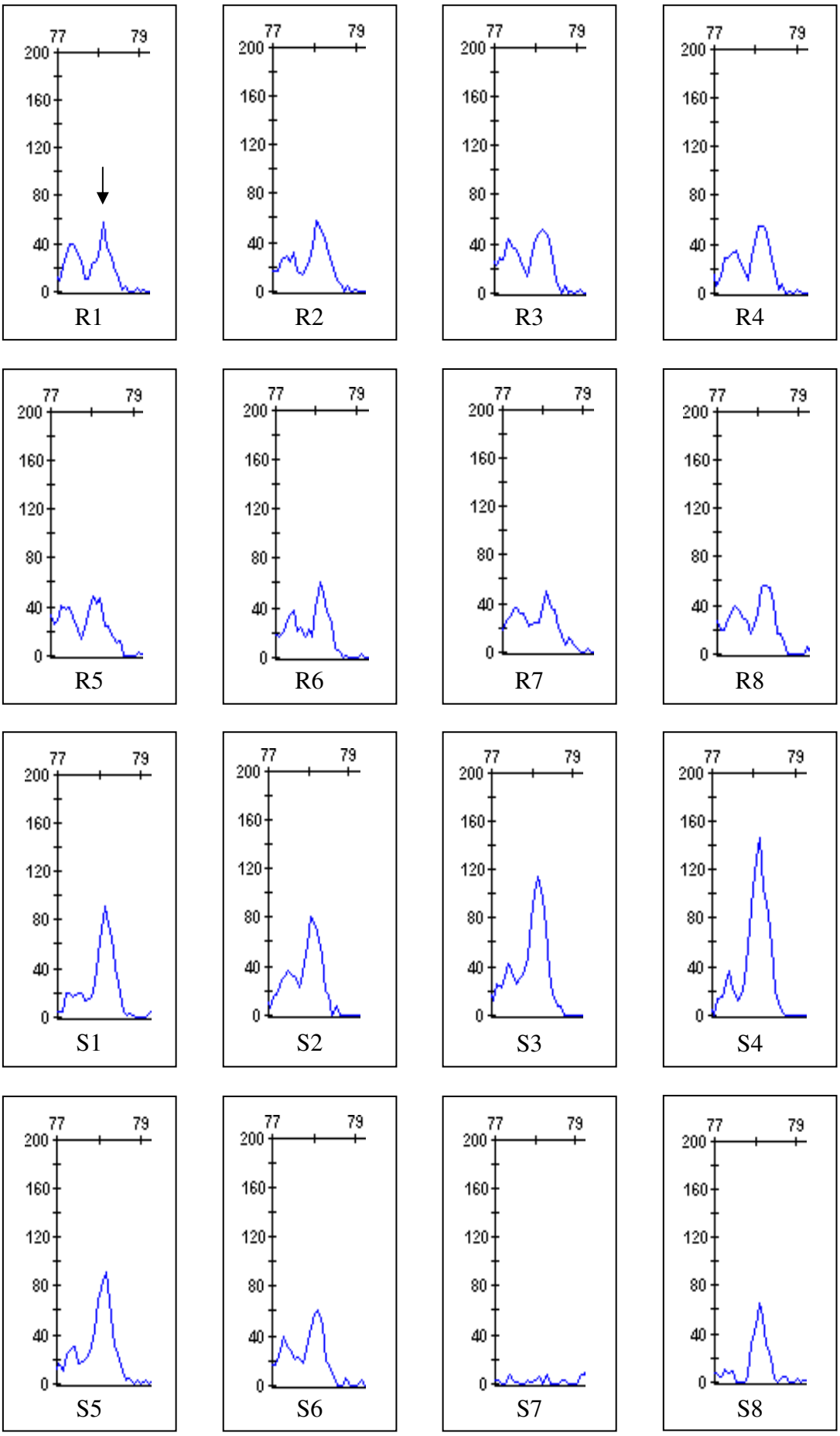

AFLP primer combination *Eco*RI-ACA/*Mse*I-CAG – polymorphic allele 314 bp



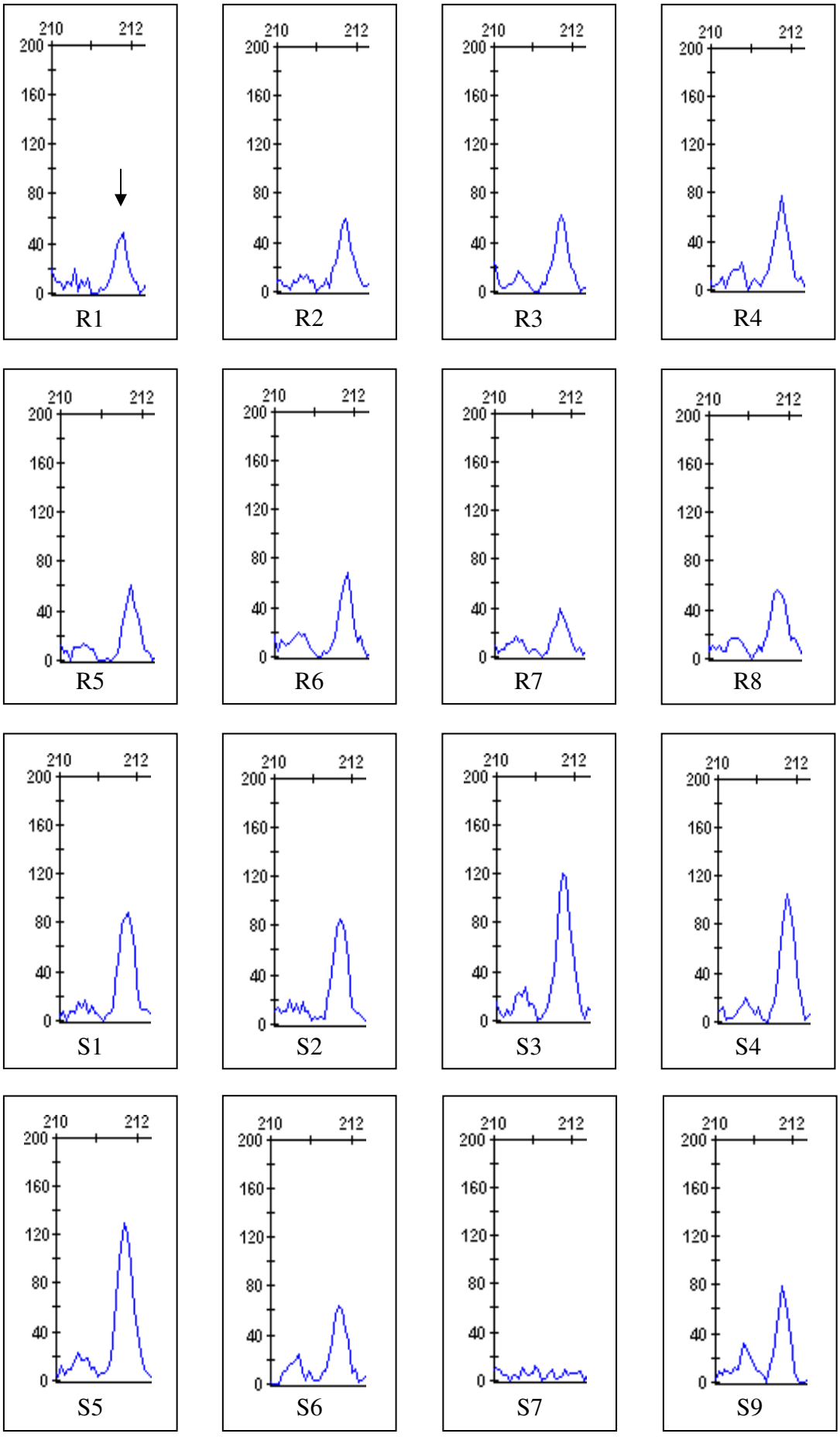

AFLP primer combination *Eco*RI-AGG/*Mse*I-CAG – polymorphic allele 212 bp



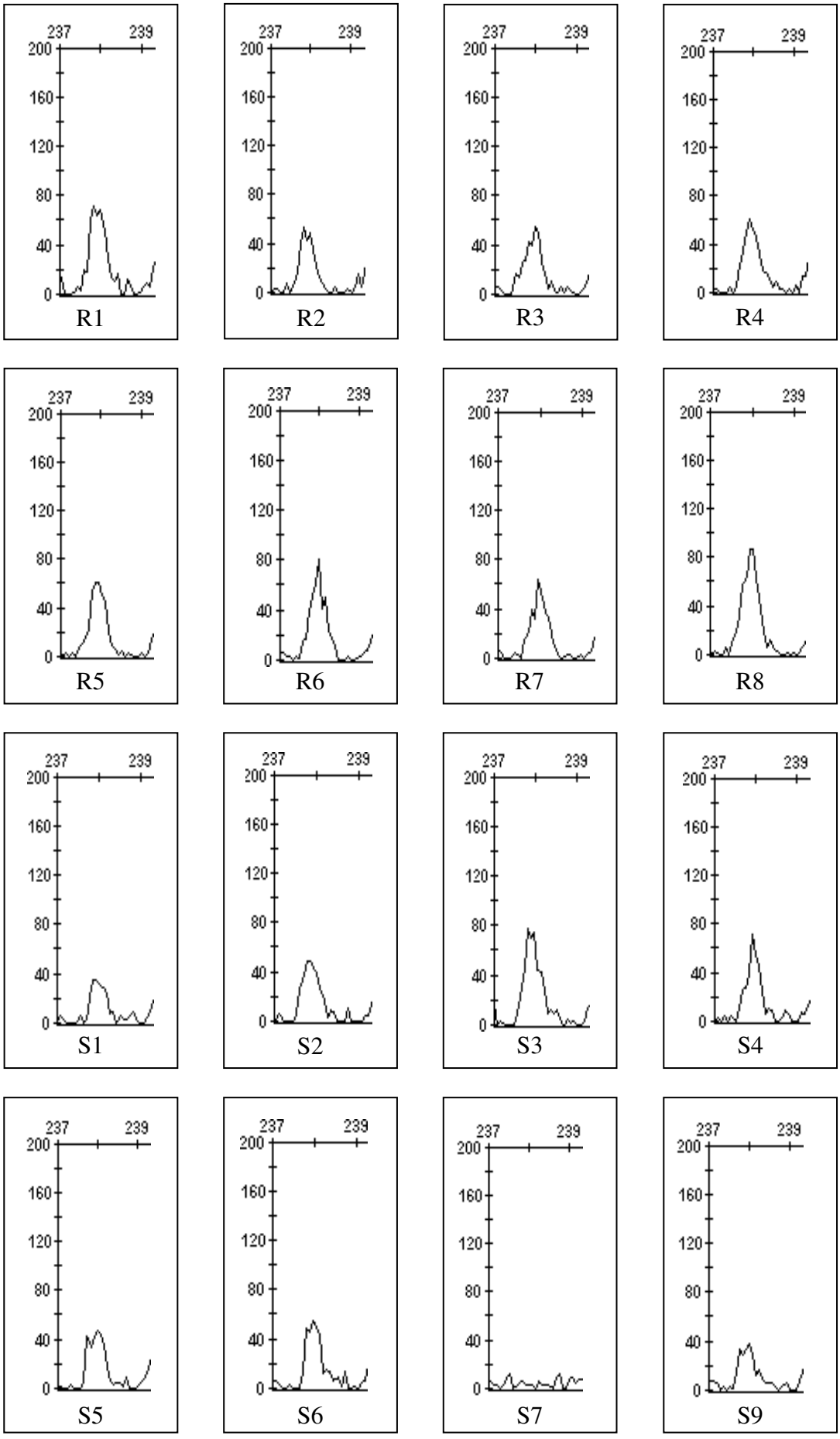

AFLP primer combination *Eco*RI-AGG/*Mse*I-CAG – polymorphic allele 228 bp



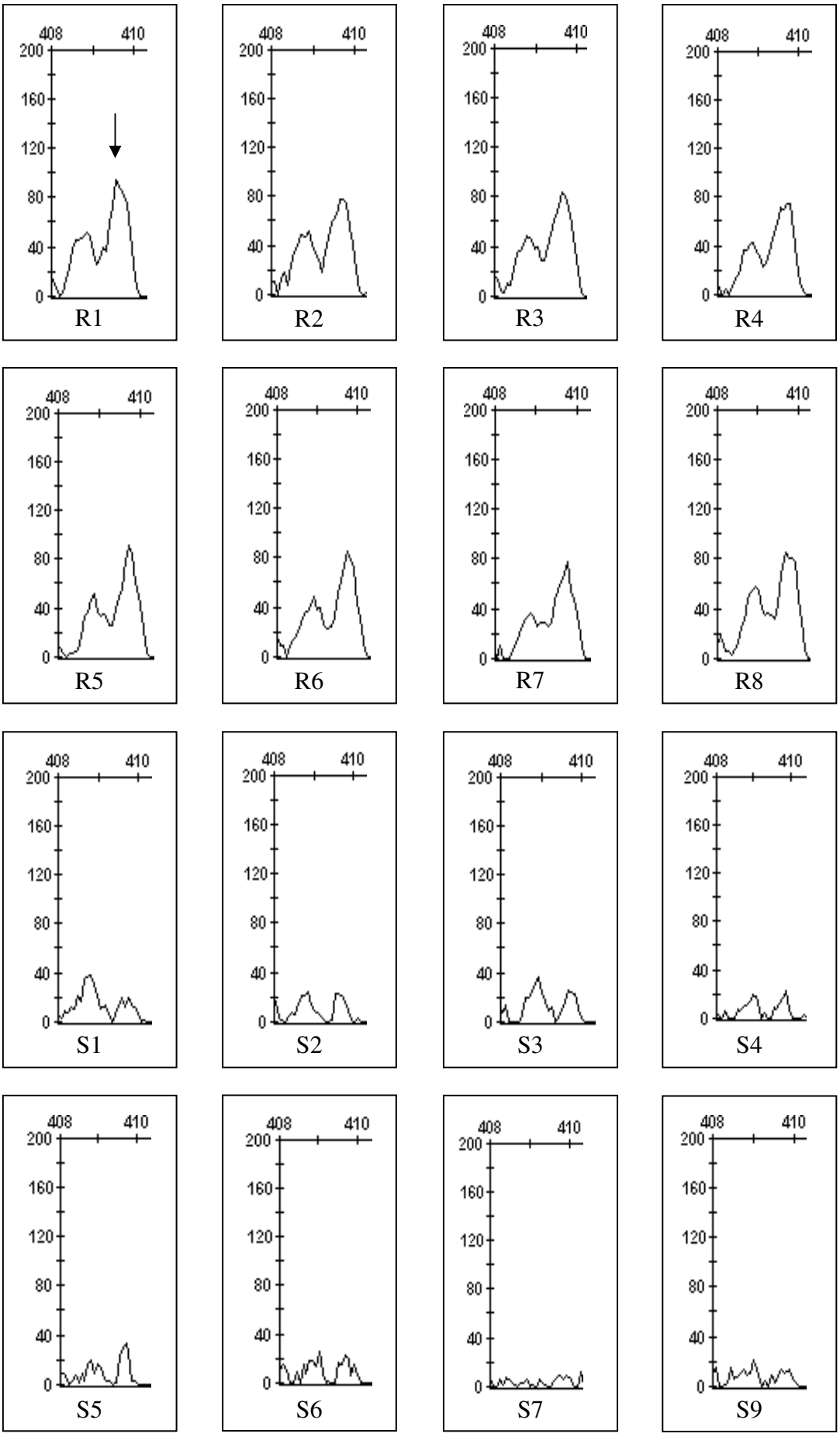

AFLP primer combination *Eco*RI-ACA/*Mse*I-CAT – polymorphic allele 78 bp



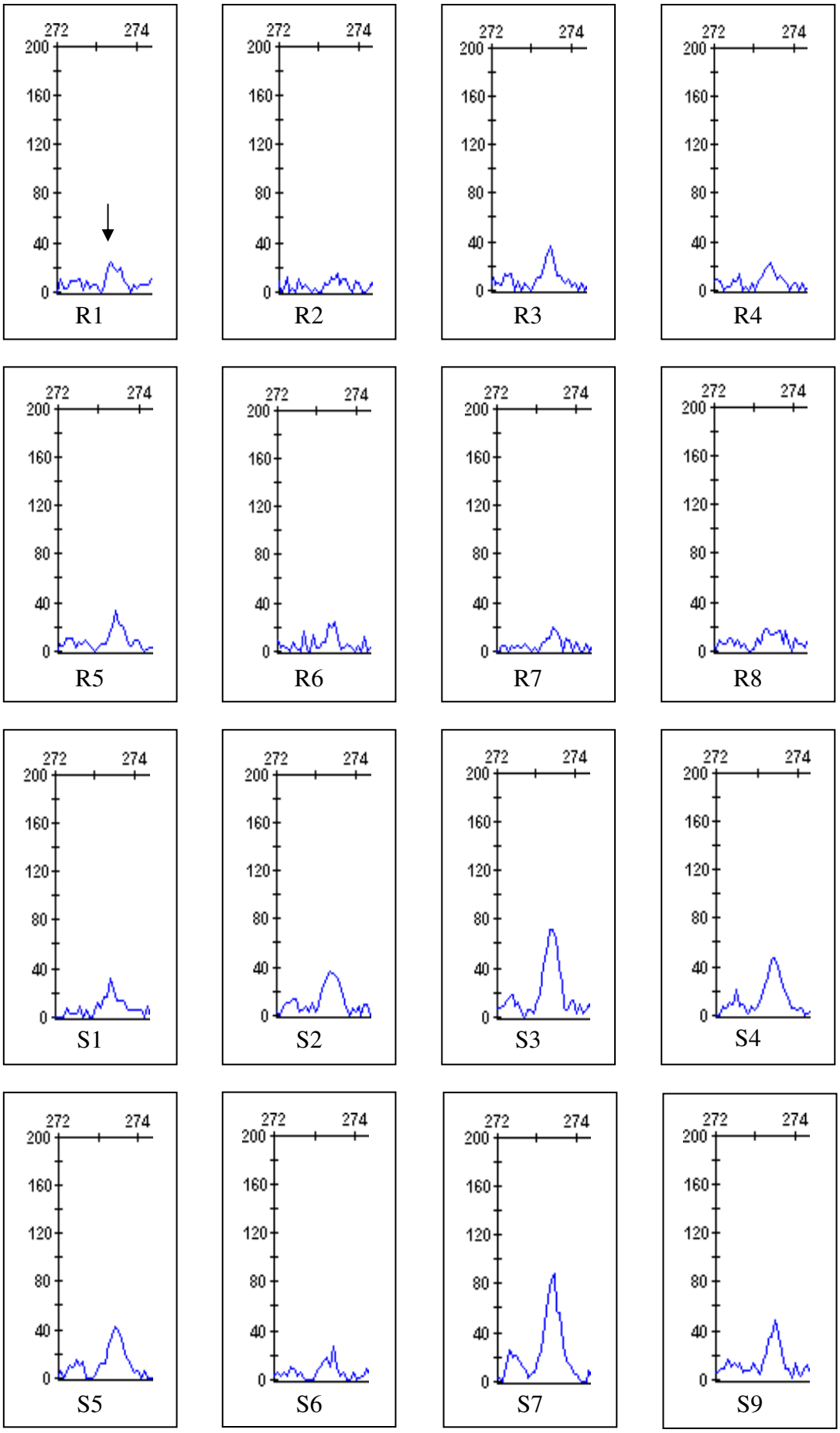

AFLP primer combination *Eco*RI-ACA/*Mse*I-CAT – polymorphic allele 212 bp



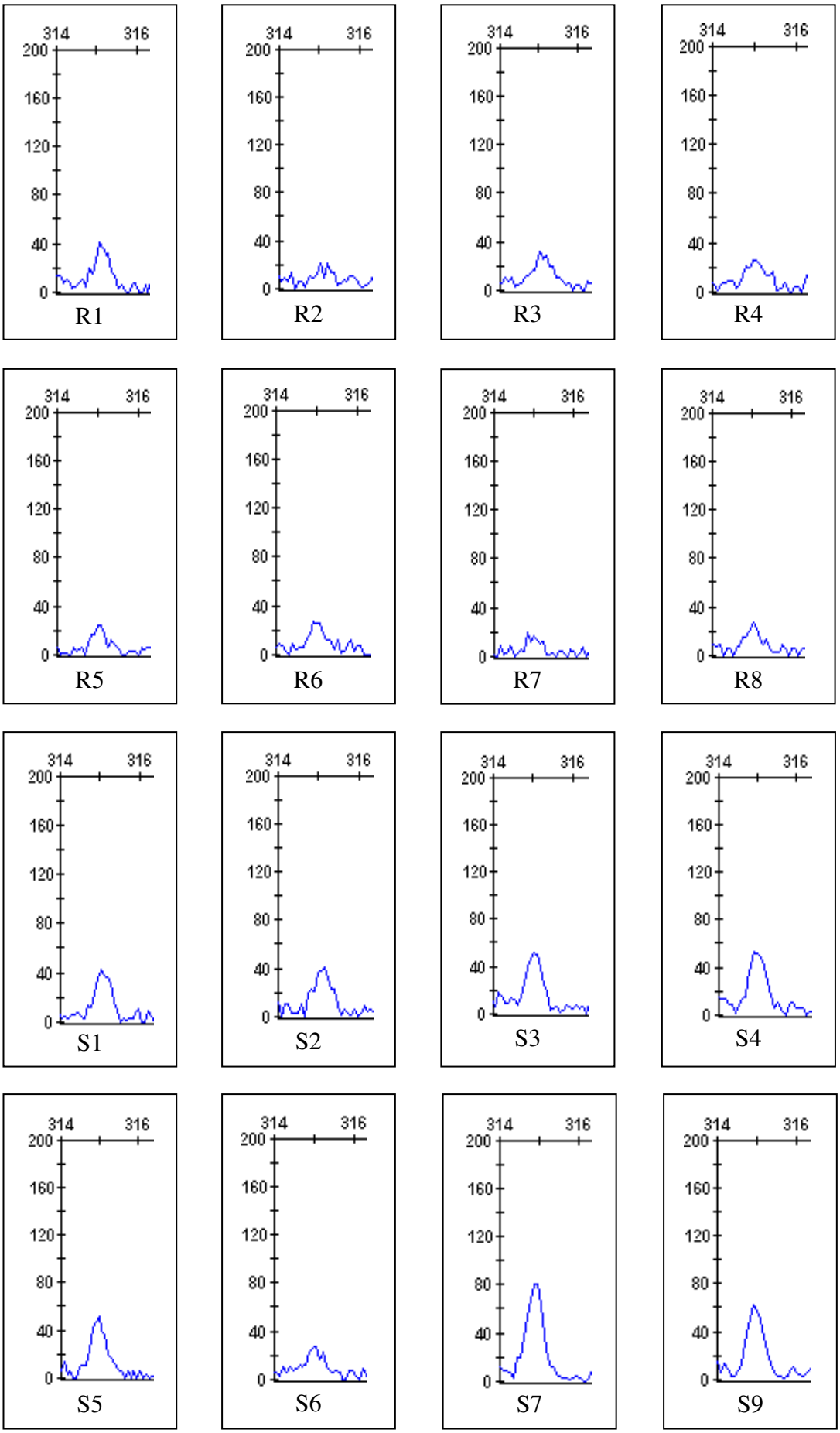

AFLP primer combination *EcoRI*-AAC/*Mse*I-CAT – polymorphic allele 238 bp



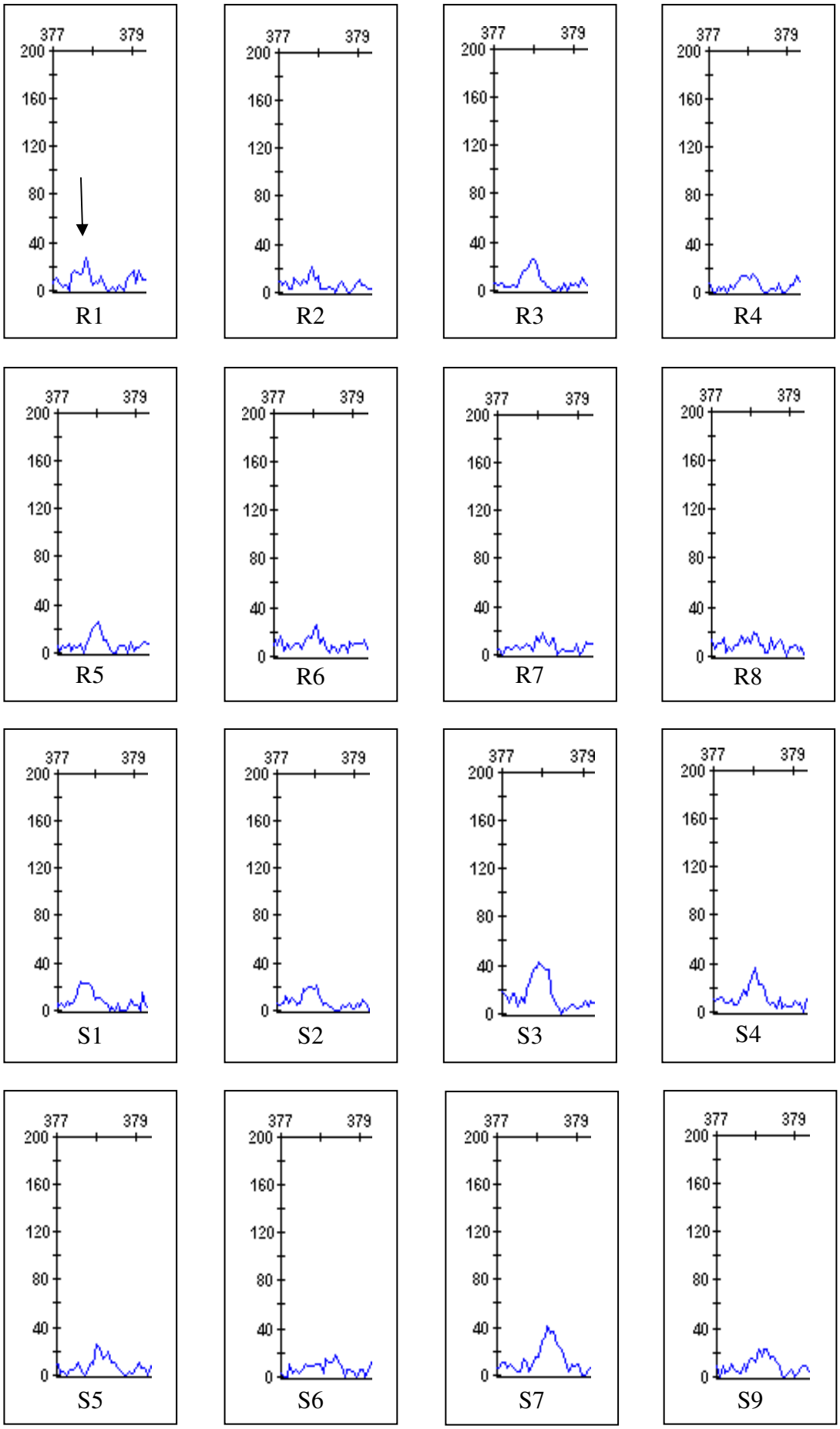

AFLP primer combination *Eco*RI-AAC/*Mse*I-CAT – polymorphic allele 410 bp



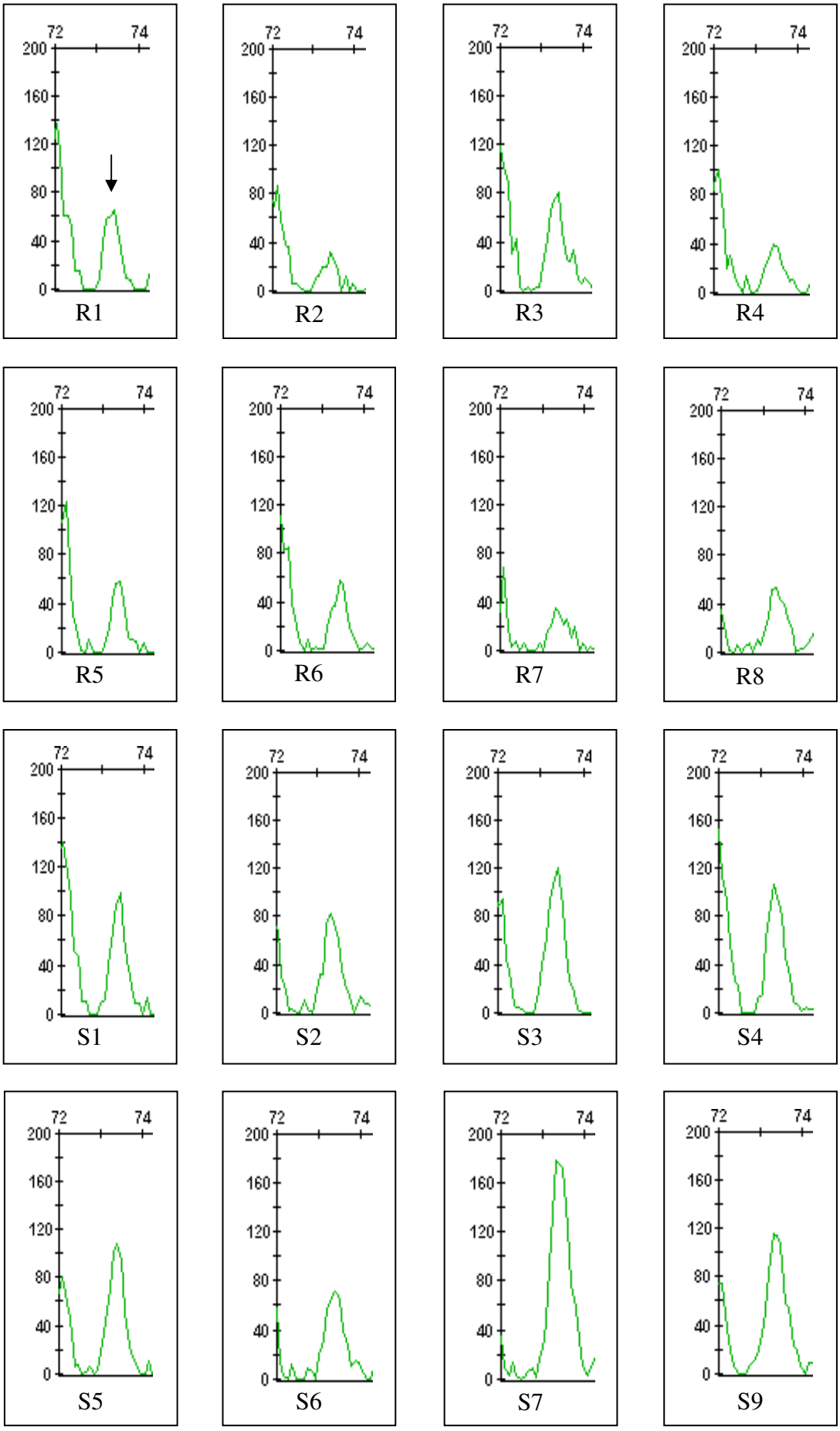

AFLP primer combination *Eco*RI - ACA/*Mse*I - CTA - polymorphic allele 273 bp



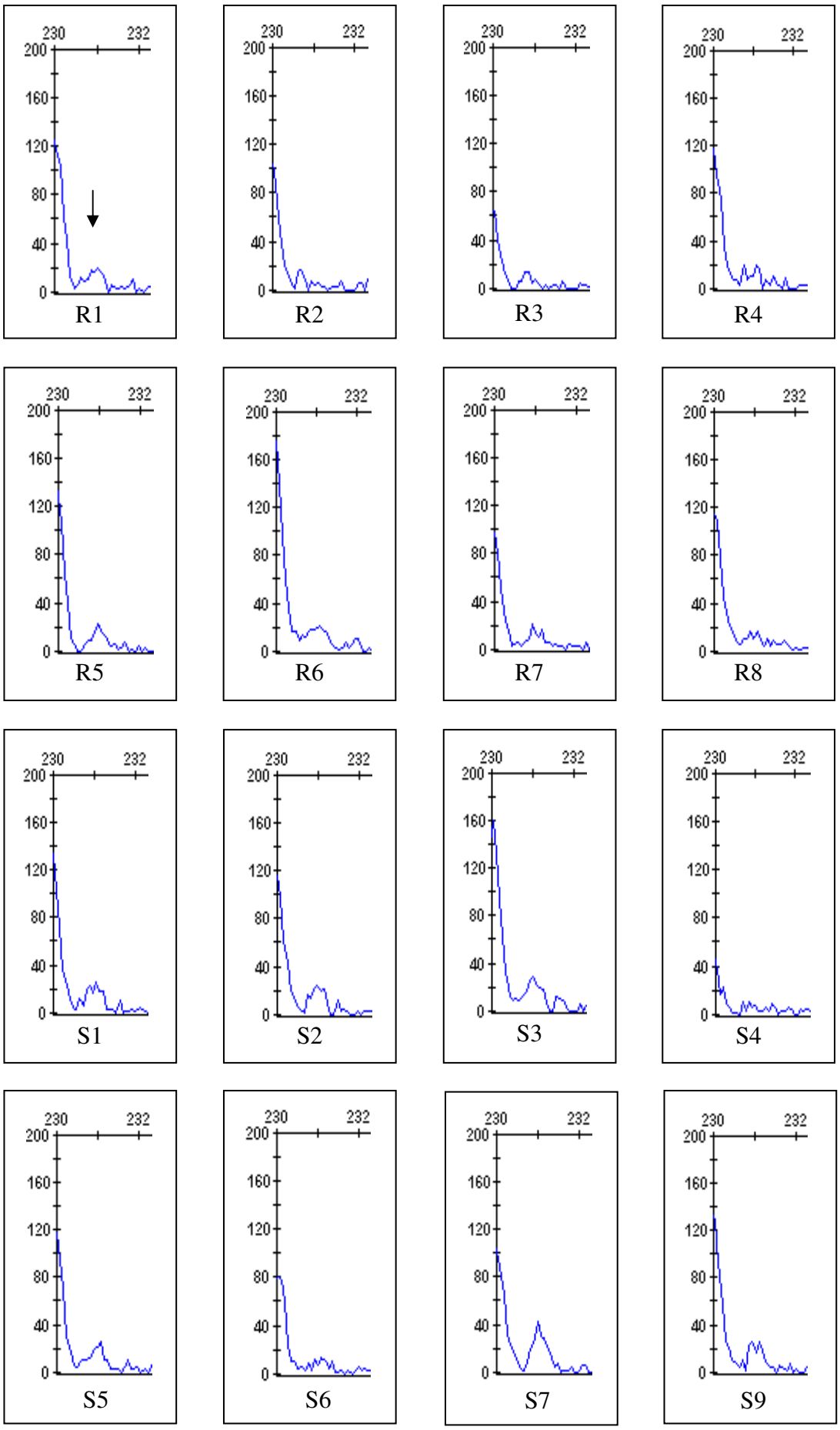

AFLP primer combination *Eco*RI - ACA/*Mse*I - CTA - polymorphic allele 315 bp



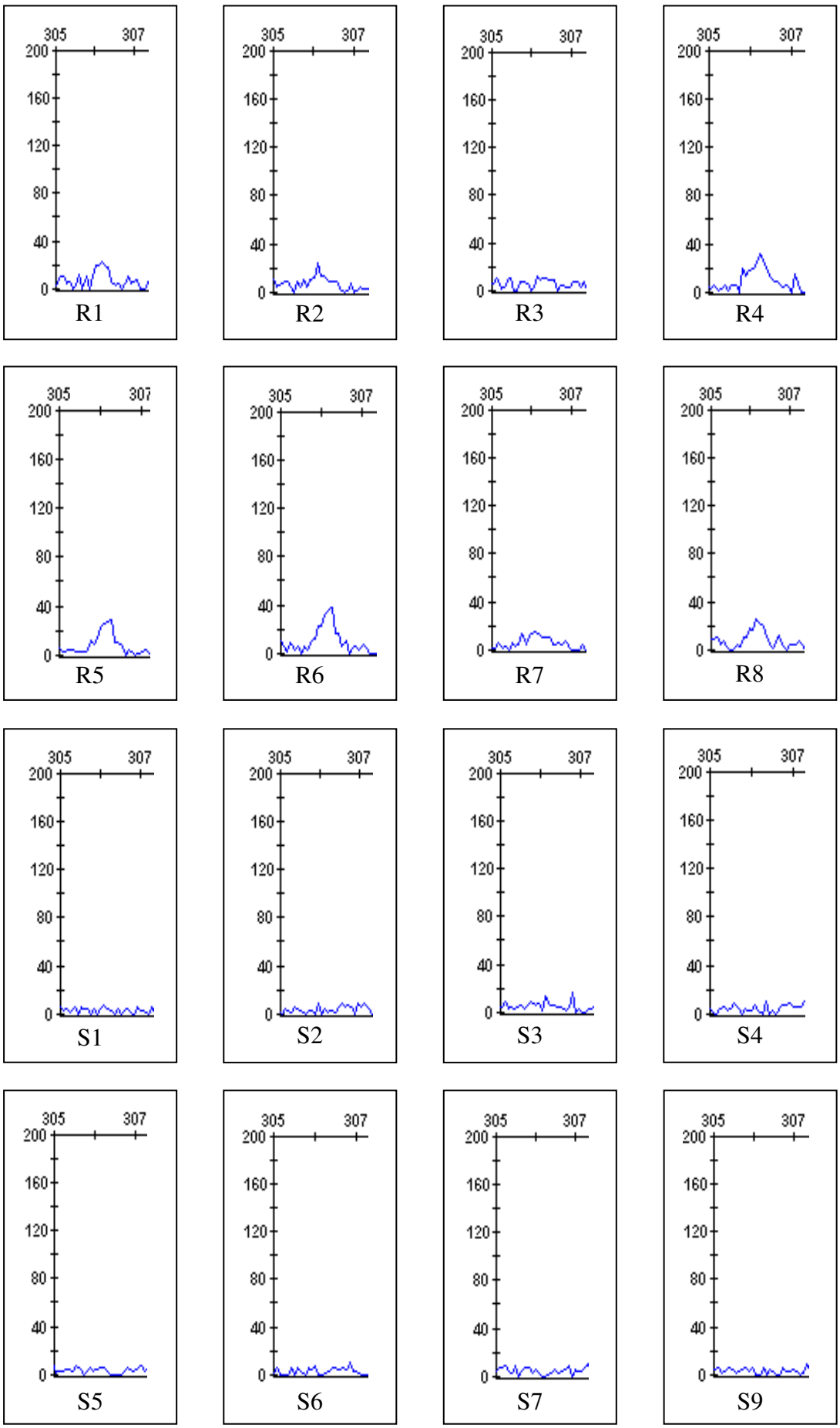

AFLP primer combination *Eco*RI - ACA/*Mse*I - CTA - polymorphic allele 378 bp



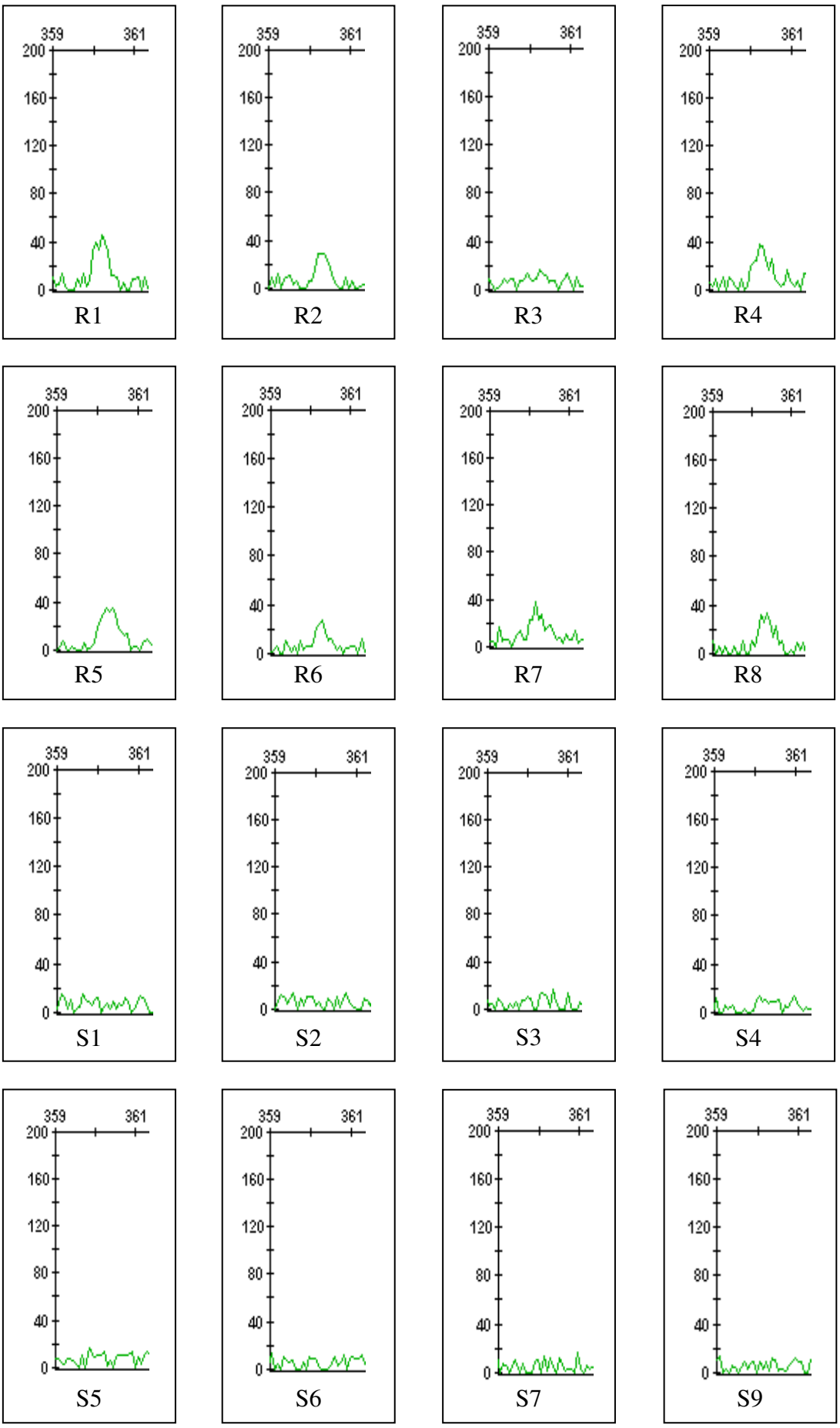

AFLP primer combination *Eco*RI-AGG/*Mse*I-CTA - polymorphic allele 73 bp



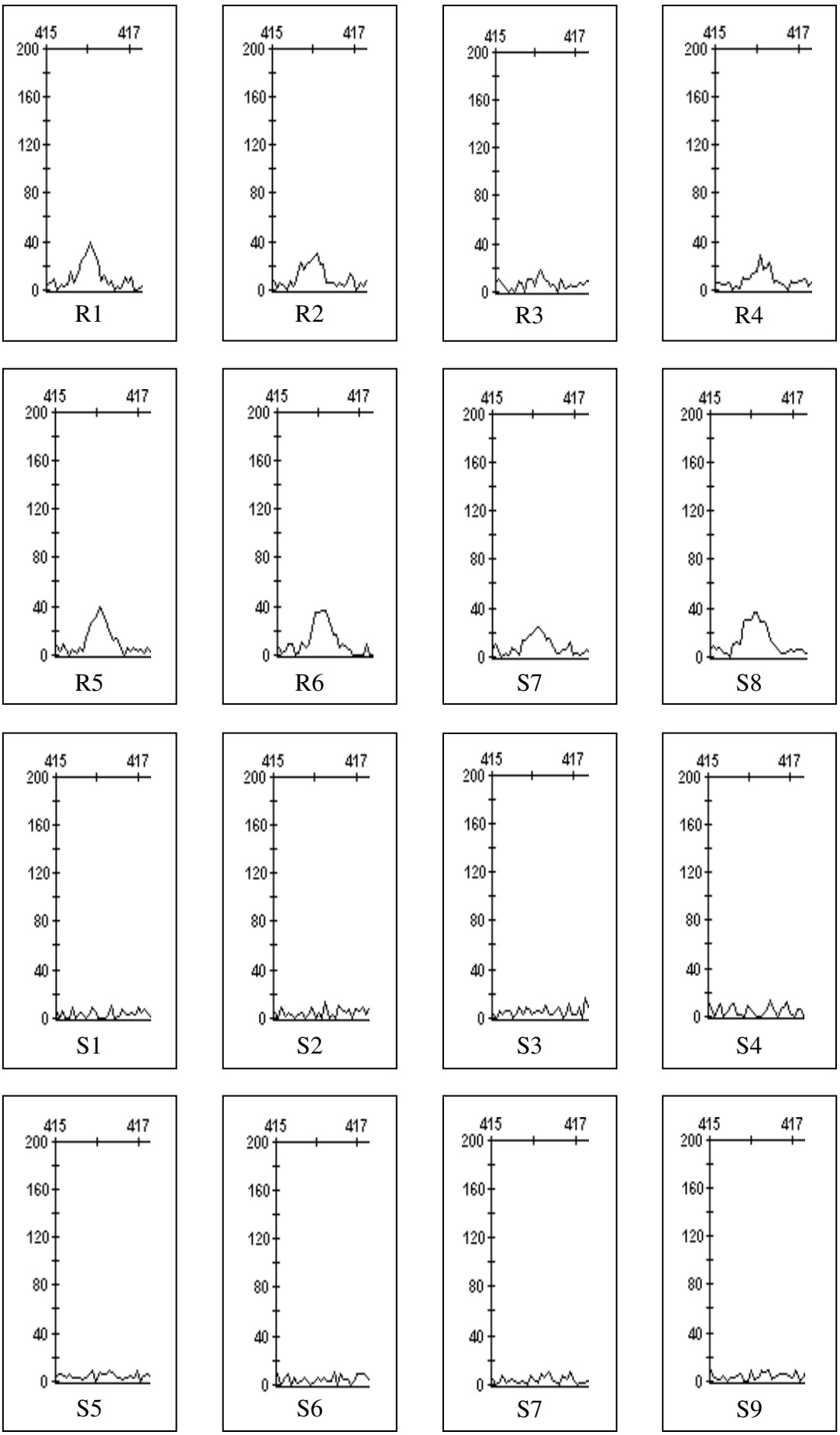

AFLP primer combination *Eco*RI - ACA/*Mse*I - CTG - polymorphic allele size 231 bp




AFLP primer combination *Eco*RI - ACA/*Mse*I - CTG - polymorphic allele 306 bp



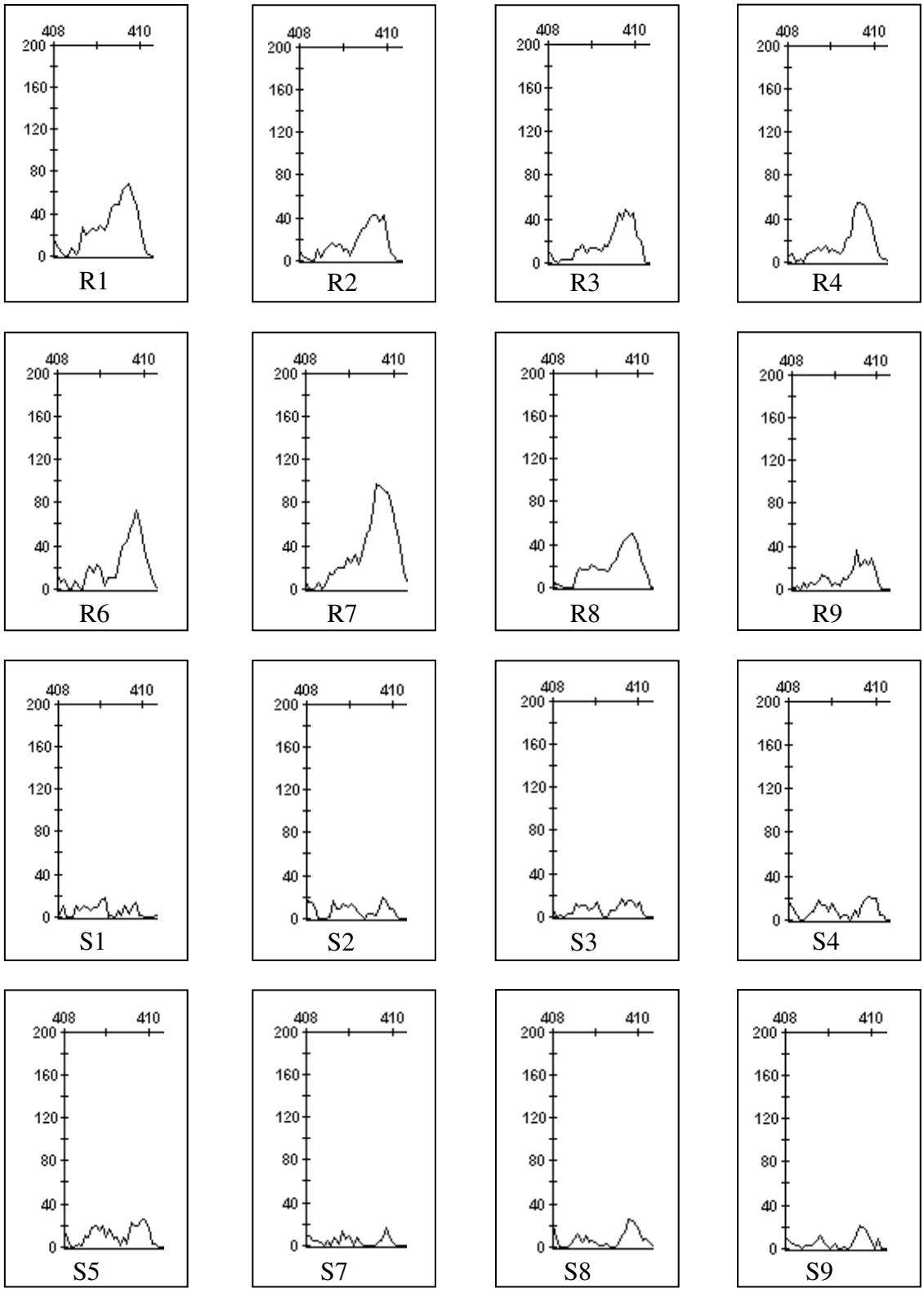

AFLP primer combination *Eco*RI - AGG/*Mse*I - CTG - polymorphic allele 360 bp




AFLP primer combination *Eco*RI - AAC/*Mse*I - CTG - polymorphic allele 416 bp

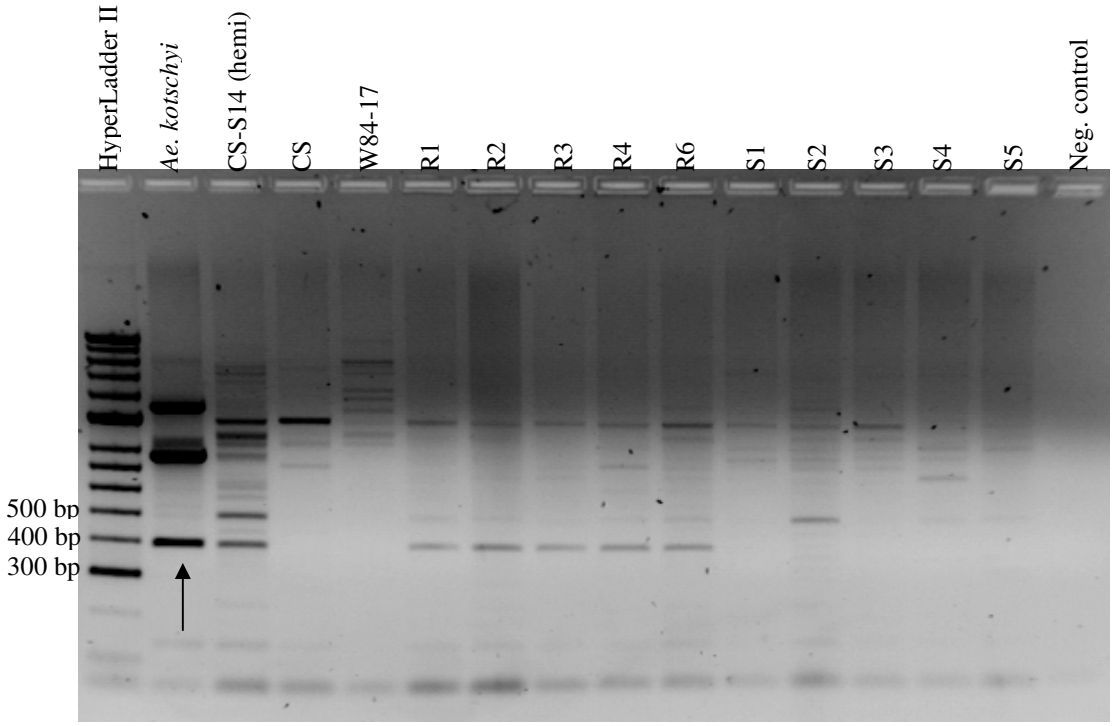
# Addendum F

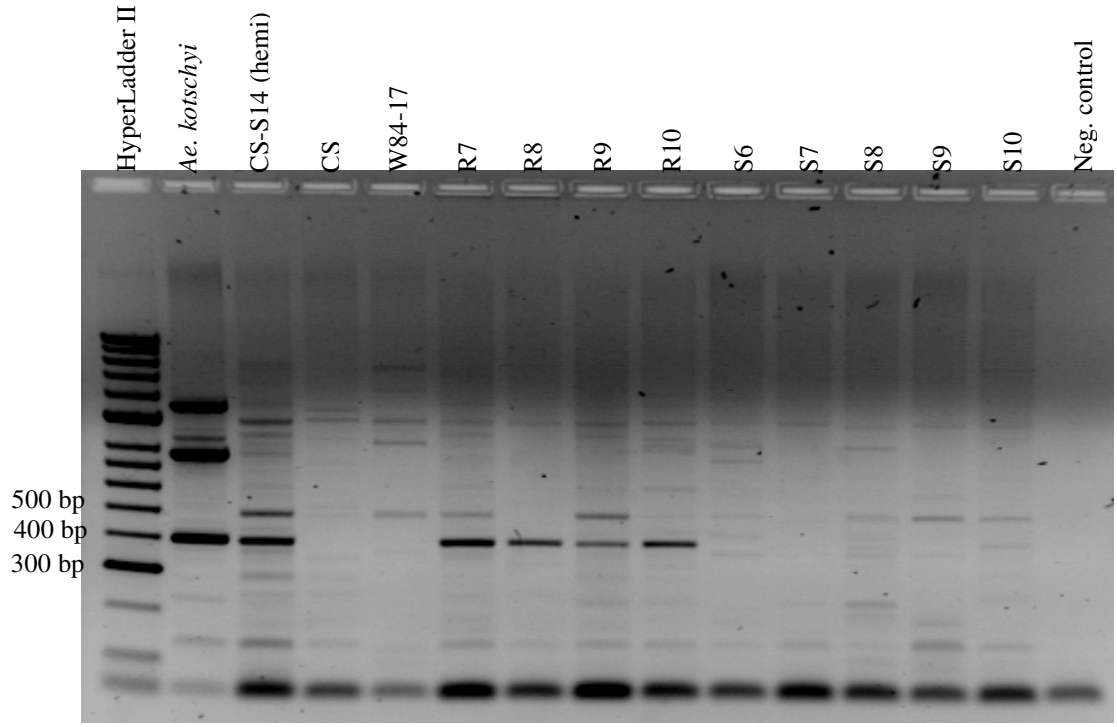
Confirmation that the *Eco*RI – AAC/*Mse*I – CAT 410 AFLP band is associated with the shortest translocation recombinant (#74). Sixteen resistant (R) and susceptible (S) $BCF_2$ progeny of recombinant #74 (cross = CS-S14 translocation/2*CS*ph1b* mutant/W84-17/3/CSN2DT2A-B/4/2*W84-17) were tested for the presence of the diagnostic band.



# Addendum G

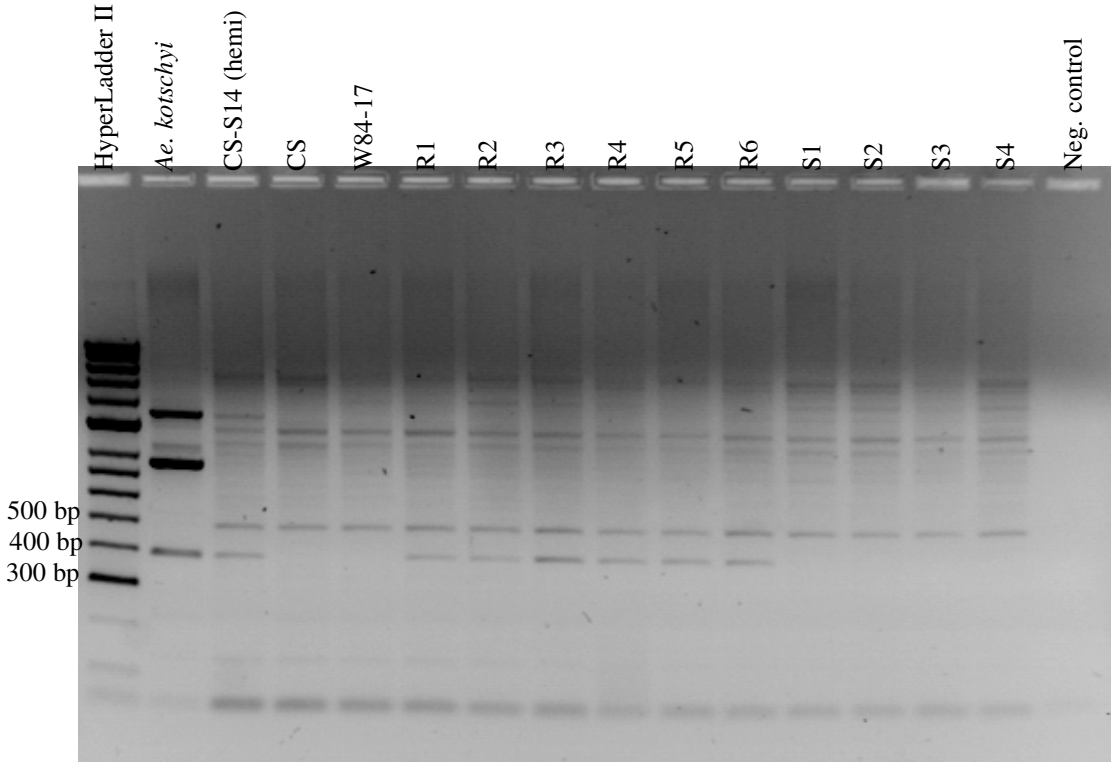
Data obtained when $BCF_2$ progeny (cross = CS-S14 translocation/2*CS*ph1b* mutant//W84-17/3/CSN2DT2A-B/4/2*W84-17) segregating for the recombined translocation #74 were tested for seedling leaf rust resistance and the presence of the *Xscar-410* locus. Resistant plants are marked as R1-R10 and susceptible plants as S1-S10. The arrow indicates the diagnostic SCAR-410 band.

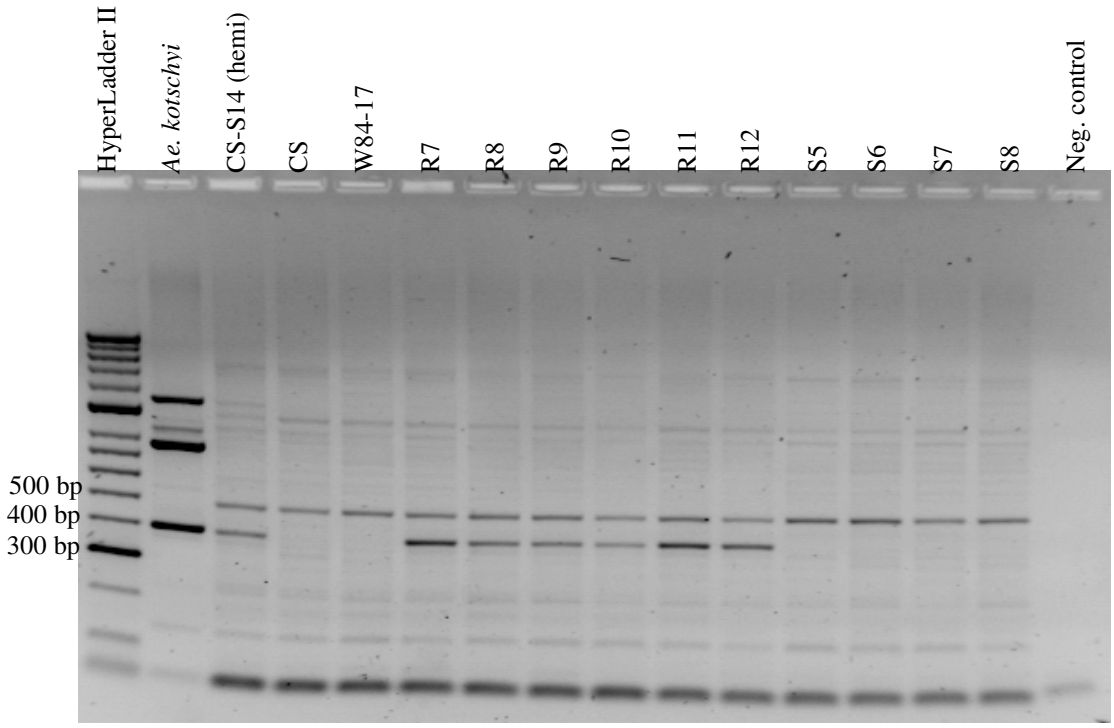


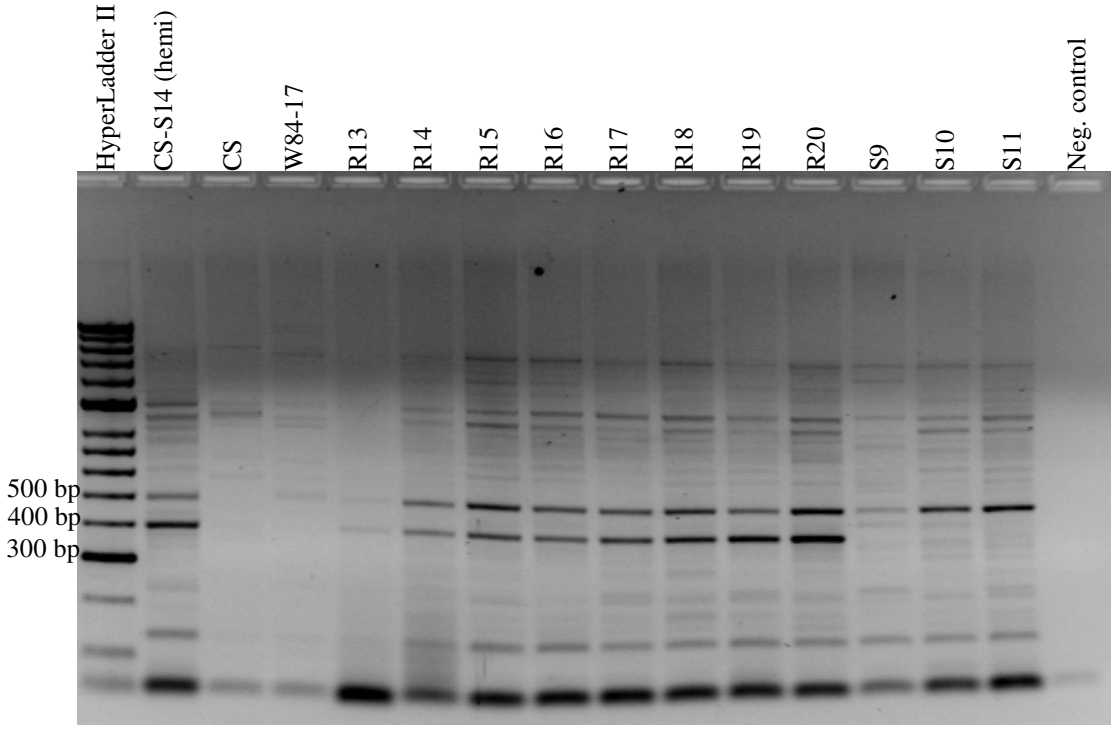



# Addendum H

Data obtained when $BCF_3$ progeny (cross = CS-S14 translocation/2*CS*ph1b* mutant//W84-17/3/CSN2DT2A-B/4/2*W84-17) segregating for the recombined translocation #74 were tested for seedling leaf rust resistance and the presence of the *Xscar-410* locus. Resistant plants are marked as R1-R20 and susceptible plants as S1-S11. The arrow indicates the diagnostic SCAR410 band.




HyperLadder II
CS-S14 (hemi)
CS
W84-17
R13
R14
R15
R16
R17
R18
R19
R20
S9
S10
S11
Neg. control
500 bp
400 bp
300 bp

# Addendum I

Measurements of days to ear emergence, days to flowering, 100 kernel mass and yield of $F_2$ plants derived from the crosses CS X $F_1$: 0514 (plant height experimental group) and CS X W84-17 (plant height control group).

## CS X $F_1$: 0514 (Experimental Group)

| Plant nr. | Plant height (cm) | Days to ear emergence | Days to flowering | 100 kernel mass (g) | Yield (g) |
|---|---|---|---|---|---|
| 1 R | 118 | 104 | 113 | 5.61 | 5.96 |
| 2 R* | 80 | 90 | 101 | 4.87 | 4.82 |
| 3 R | 85 | 106 | 115 | 5.32 | 5.77 |
| 4 S | 118 | 80 | 88 | 5.22 | 50.26 |
| 5 R | 160 | 97 | 105 | 6.05 | 24.09 |
| 6S | 82 | 110 | 120 | 2.79 | 3.95 |
| 7 R | 55 | 116 | / | Sterile | Sterile |
| 8 R* | 80 | 108 | 120 | 3.89 | 1.48 |
| 9 R | 125 | 101 | 110 | 5.38 | 4.84 |
| 10 R | 174 | 103 | 113 | 5.18 | 33.08 |
| 11 R | 157 | 101 | 110 | 5.73 | 45.78 |
| 12 R* | 79 | 99 | 110 | 5.13 | 3.08 |
| 13 R* | 68 | 99 | 109 | 6.00 | 0.06 |
| 14 R | 144 | 97 | 105 | 5.58 | 19.41 |
| 15 S | 153 | 95 | 104 | 5.45 | 42.17 |
| 16R* | 84 | 88 | 100 | 5.37 | 1.02 |
| 17 S | 120 | 65 | 76 | 4.51 | 17.16 |
| 18 R | 114 | 75 | 85 | 4.97 | 20.77 |
| 19 S | 127 | 62 | 72 | 4.74 | 12.07 |
| 20 R | 112 | 70 | 80 | 5.82 | 15.46 |
| 21 S | 103 | 64 | 74 | 3.90 | 9.52 |
| 22 R | 94 | 100 | 110 | 4.10 | 41.55 |
| 23 R* | 60 | 89 | 99 | 4.90 | 1.52 |
| 24 R* | 98 | 100 | 111 | 4.82 | 3.71 |
| 25 R | 147 | 87 | 96 | 5.68 | 29.38 |
| 26 S | 138 | 108 | 119 | 3.79 | 44.94 |
| 27 R | 133 | 87 | 98 | 5.02 | 12.86 |
| 28 R* | 59 | 101 | / | 4.50 | 2.88 |
| 29 R* | 69 | 86 | 97 | 3.69 | 1.18 |
| 30 R | 96 | 82 | 93 | 4.45 | 11.33 |
| 31 R* | 90 | 84 | 97 | 5.17 | 0.31 |
| 32 R* | 75 | 92 | 103 | 4.73 | 0.71 |
| 33 R* | 113 | 83 | 94 | 5.56 | 1.78 |
| 34 R | 82 | 81 | 91 | 4.97 | 1.49 |
| 35 R | 135 | 81 | 92 | 5.22 | 37.59 |
| 36 R | 129 | 84 | 95 | 4.81 | 13.67 |
| 37 R | 132 | 85 | 94 | 5.49 | 24.27 |

Continue.

| Plant nr. | Plant height (cm) | Days to ear emergence | Days to flowering | 100 kernel mass (g) | Yield (g) |
|---|---|---|---|---|---|
| 38 R* | 48 | 71 | 81 | 4.25 | 0.17 |
| 39 S | 142 | 72 | 80 | 5.80 | 20.78 |
| 40 R | 147 | 94 | 104 | 6.10 | 27.16 |
| 41 R* | 82 | 99 | 111 | 4.60 | 0.69 |
| 42 R | 120 | 77 | 89 | 4.46 | 20.07 |
| 43 R | 134 | 89 | 99 | 5.71 | 19.4 |
| 44 R | 131 | 81 | 92 | 5.03 | 24.64 |
| 45 R* | 88 | 91 | 102 | 4.40 | 0.22 |
| 46 R* | 95 | 88 | 98 | 4.61 | 1.29 |
| 47 S | 167 | 117 | 124 | 4.88 | 66.25 |
| 48 R | 110 | 116 | 124 | 4.93 | 36.30 |
| 49 R | 133 | 115 | 124 | 4.56 | 10.51 |
| 50 R | 112 | 144 | / | 4.36 | 8.56 |
| 51 S | 154 | 79 | 88 | 5.22 | 40.20 |
| 52 R* | 115 | 101 | 110 | 5.58 | 1.34 |
| 53 R | 111 | 95 | 104 | 5.70 | 16.15 |
| 54 R | 143 | 79 | 88 | 6.04 | 22.24 |
| 55 R | 157 | 91 | 102 | 5.83 | 42.05 |
| 56 R | 116 | 64 | 74 | 5.81 | 13.19 |
| 57 R | 110 | 72 | 82 | 5.58 | 19.84 |
| 58 S | 104 | 57 | 69 | 4.81 | 17.06 |
| 59 R | 135 | 84 | 94 | 5.83 | 31.25 |
| 60 R | 110 | 73 | 82 | 6.4 | 13.13 |
| 61 S | 165 | 91 | 100 | 5.48 | 33.76 |
| 62 R | 147 | 93 | 103 | 5.42 | 15.71 |
| 63 R | 143 | 95 | 104 | 5.26 | 12.13 |
| 64 R | 158 | 81 | 91 | 6.98 | 41.10 |
| 65 R | 150 | 99 | 109 | 5.12 | 11.93 |
| 66 R | 128 | 81 | 91 | 5.43 | 16.53 |
| 67 R* | 72 | 78 | 88 | 3.94 | 0.67 |
| 68 R | 86 | 73 | 81 | 5.76 | 8.20 |
| 69 R | 114 | 65 | 76 | 3.95 | 13.38 |
| 70 R | 123 | 79 | 88 | 5.99 | 17.54 |
| 71 R* | 76 | 109 | 120 | 5.13 | 0.41 |
| 72 R* | 101 | 113 | 122 | 4.85 | 2.67 |
| 73 R* | 83 | 90 | 98 | 4.28 | 1.97 |
| 74 R* | 70 | 59 | 72 | 3.14 | 0.69 |
| 75 R | 173 | 98 | 108 | 6.23 | 38.93 |
| 76 S | 121 | 81 | 91 | 5.24 | 26.21 |
| 77 R* | 81 | 73 | 78 | 4.84 | 3.68 |
| 78 S | 158 | 98 | 107 | 5.03 | 39.85 |
| 79 R* | 70 | 102 | 115 | 5.80 | 0.58 |
| 80 R | 92 | 96 | 108 | 5.96 | 13.55 |
| 81 R | 148 | 88 | 97 | 5.83 | 38.63 |

Continue.

| Plant nr. | Plant height (cm) | Days to ear emergence | Days to flowering | 100 kernel mass (g) | Yield (g) |
|---|---|---|---|---|---|
| 82 R* | 59 | / | / | 3.00 | 0.09 |
| 83 S* | 81 | 110 | 126 | 5.54 | 4.49 |
| 84 R* | 80 | 104 | 117 | 4.50 | 0.63 |
| 85 R* | 93 | 92 | 103 | 5.35 | 1.39 |
| 86 R* | 70 | 102 | 114 | 4.95 | 0.99 |
| 87 R* | 87 | 85 | 97 | 4.85 | 0.63 |
| 88 R* | 81 | 97 | 108 | 6.00 | 0.06 |
| 89 R* | 104 | 113 | 122 | 4.65 | 1.44 |
| 90 R* | 62 | 105 | 119 | 3.54 | 0.92 |

## CS X W84-17 (Control Group)

| Plant nr. | Plant height (cm) | Days to ear emergence | Days to flowering | 100 kernel mass (g) | Yield (g) |
|---|---|---|---|---|---|
| 1 | 121 | 76 | 82 | 3.97 | 4.04 |
| 2 | 137 | 98 | 107 | 4.00 | 31.89 |
| 3 | 109 | 97 | 105 | 4.12 | 13.81 |
| 4 | 123 | 89 | 97 | 5.83 | 8.38 |
| 5 | 144 | 90 | 101 | 5.38 | 48.18 |
| 6 | 135 | 96 | 105 | 4.95 | 29.32 |
| 7 | 108 | 93 | 104 | 4.91 | 12.61 |
| 8 | 133 | 61 | 72 | 6.31 | 25.98 |
| 9 | 93 | 59 | 71 | 4.66 | 18.21 |
| 10 | 117 | 72 | 83 | 4.36 | 31.64 |
| 11 | 105 | 103 | 110 | 3.16 | 24.01 |
| 12 | 170 | 85 | 96 | 5.68 | 55.74 |
| 13 | 156 | 91 | 103 | 5.21 | 45.59 |
| 14 | 96 | 83 | 95 | 5.16 | 10.24 |
| 15 | 159 | 91 | 101 | 5.36 | 69.72 |
| 16 | 121 | 79 | 90 | 4.84 | 21.83 |
| 17 | 97 | 60 | 71 | 5.05 | 12.88 |
| 18 | 157 | 100 | 110 | 3.04 | 28.94 |
| 19 | 156 | 102 | 112 | 4.38 | 32.02 |
| 20 | 142 | 87 | 97 | 5.65 | 40.11 |
| 21 | 166 | 91 | 100 | 5.44 | 45.06 |
| 22 | 146 | 85 | 94 | 5.07 | 23.56 |
| 23 | 138 | 116 | 125 | 4.70 | 50.36 |
| 24 | 155 | 122 | 131 | 4.32 | 34.55 |
| 25 | 92 | 65 | 75 | 3.52 | 6.56 |
| 26 | 134 | 78 | 89 | 4.97 | 42.85 |
| 27 | 99 | 77 | 88 | 4.33 | 23.18 |

Continue.

| Plant nr. | Plant height (cm) | Days to ear emergence | Days to flowering | 100 kernel mass (g) | Yield (g) |
|---|---|---|---|---|---|
| 28 | 174 | 99 | 110 | 5.50 | 29.12 |
| 29 | 120 | 88 | 100 | 4.06 | 23.74 |
| 30 | 143 | 66 | 78 | 6.05 | 34.72 |
| 31 | 139 | 70 | 81 | 4.34 | 26.82 |
| 32 | 133 | 92 | 102 | 6.15 | 36.47 |
| 33 | 132 | 81 | 91 | 4.52 | 56.15 |
| 34 | 127 | 62 | 73 | 5.08 | 40.17 |
| 35 | 98 | 56 | 67 | 2.69 | 6.58 |
| 36 | 178 | 92 | 102 | 5.48 | 56.78 |
| 37 | 151 | 89 | 99 | 5.78 | 45.79 |
| 38 | 118 | 114 | 125 | 4.01 | 34.54 |
| 39* | 107 | 114 | 124 | 4.89 | 3.91 |
| 40 | 99 | 76 | 80 | 4.86 | 14.66 |

*The 100 kernel mass was calculated using less than one hundred seeds